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<b>(21) International Application Number:</b> PCT/US95/07536 <b>(22) International Filing Date:</b> 15 June 1995 (15.06.95)  <b>(30) Priority Data:</b> 08/265,534      24 June 1994 (24.06.94)      US 08/441,147      15 May 1995 (15.05.95)      US  <b>(71) Applicants:</b> NOVO NORDISK BIOTECH, INC. [US/US]; 1445 Drew Avenue, Davis, CA 95616-4880 (US). NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).  <b>(72) Inventors:</b> YAVER, Debbie, Sue; 2809 Albany Avenue, Davis, CA 95616 (US). XU, Feng; 1534 Carmel Valley Drive, Woodland, CA 95776 (US). DALBØGE, Henrik; Parkvej 28, DK-2830 Virum (DK). SCHNEIDER, Palle; Rydtoften 43, DK-2750 Ballerup (DK). AASLYNG, Dorrit, Anita; Gartnerkrogen 69, DK-3500 Værløse (DK).  <b>(74) Agents:</b> ZELSON, Steve, T. et al.; Novo Nordisk of North America, Inc., Suite 6400, 405 Lexington Avenue, New York, NY 10174 (US).		<b>(81) Designated States:</b> AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> PURIFIED POLYPORUS LACCASES AND NUCLEIC ACIDS ENCODING SAME		
<b>(57) Abstract</b>  The present invention relates to isolated nucleic acid constructs containing a sequence encoding a <i>Polyporus</i> laccase, and the laccase proteins encoded thereby.		

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PURIFIED *POLYPORUS* LACCASES AND NUCLEIC ACIDS  
ENCODING SAME

5

Field of the Invention

The present invention relates to isolated nucleic acid fragments encoding a fungal oxidoreductase enzyme and the  
10 purified enzymes produced thereby. More particularly, the invention relates to nucleic acid fragments encoding a phenol oxidase, specifically a laccase, of a basidiomycete, *Polyporus*.

15 Background of the Invention

Laccases (benzenediol:oxygen oxidoreductases) are multi-copper-containing enzymes that catalyze the oxidation of phenolics. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable  
20 phenolic substrate; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Such reactions are important in nature in biosynthetic pathways which lead to the formation of melanin, alkaloids, toxins, lignins, and  
25 humic acids. Laccases are produced by a wide variety of fungi, including ascomycetes such as *Aspergillus*, *Neurospora*, and *Podospora*, the deuteromycete *Botrytis*, and basidiomycetes such as *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, *Polyporus* and perfect forms of *Rhizoctonia*.  
30 Laccases exhibit a wide range of substrate specificity, and each different fungal laccase usually differs only quantitatively from others in its ability to oxidize phenolic substrates. Because of the substrate diversity, laccases generally have found many potential industrial

applications. Among these are lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, juice manufacture, phenol resin production, and waste water treatment.

- 5        Although the catalytic capabilities are similar, laccases made by different fungal species do have different temperature and pH optima, and these may also differ depending on the specific substrate. A number of these fungal laccases have been isolated, and the genes for
- 10 several of these have been cloned. For example, Choi et al. (Mol. Plant-Microbe Interactions 5: 119-128, 1992) describe the molecular characterization and cloning of the gene encoding the laccase of the chestnut blight fungus, *Cryphonectria parasitica*. Kojima et al. (J. Biol. Chem.
- 15 265: 15224-15230, 1990; JP 2-238885) provide a description of two allelic forms of the laccase of the white-rot basidiomycete *Coriolus hirsutus*. Germann and Lerch (Experientia 41: 801, 1985; PNAS USA 83: 8854-8858, 1986) have reported the cloning and partial sequencing of the
- 20 *Neurospora crassa* laccase gene. Saloheimo et al. (J. Gen. Microbiol. 137: 1537-1544, 1985; WO 92/01046) have disclosed a structural analysis of the laccase gene from the fungus *Phlebia radiata*.

- Attempts to express laccase genes in heterologous
- 25 fungal systems frequently give very low yields (Kojima et al., *supra*; Saloheimo et al., Bio/Technol. 9: 987-990, 1991). For example, heterologous expression of *Phlebia radiata* laccase in *Trichoderma reesei* gave only 20 mg per liter of active enzyme in lab-scale fermentation (Saloheimo,
- 30 1991, *supra*). Although laccases have great commercial potential, the ability to express the enzyme in significant quantities is critical to their commercial utility. Previous attempts to express basidiomycete laccases in recombinant hosts have resulted in very low yields. The



present invention now provides novel basidiomycete laccases which are well expressed in *Aspergillus*.

#### Summary of the Invention

5       The present invention relates to a DNA construct containing a nucleic acid sequence encoding a *Polyporus* laccase. The invention also relates to an isolated laccase encoded by the nucleic acid sequence. Preferably, the laccase is substantially pure. By "substantially pure" is  
10       meant a laccase which is essentially (i.e., ≥90%) free of other non-laccase proteins.

          In order to facilitate production of the novel laccase, the invention also provides vectors and host cells comprising the claimed nucleic acid sequence, which vectors  
15       and host cells are useful in recombinant production of the laccase. The sequence is operably linked to transcription and translation signals capable of directing expression of the laccase protein in the host cell of choice. A preferred host cell is a fungal cell, most preferably of the genus  
20       *Aspergillus*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the construct of the invention, or progeny thereof, under conditions suitable for expression of the laccase protein, and recovering the laccase protein from  
25       the culture.

          The laccases of the present invention are useful in a number of industrial processes in which oxidation of phenolics is required. These processes include lignin manipulation, juice manufacture, phenol polymerization and  
30       phenol resin production.

#### Brief Description of the Figures

          Figure 1 shows the DNA sequence and translation of genomic clone 21GEN, containing LCC1 (SEQ ID NO. 1)

Figure 2 shows the DNA sequence and translation of genomic clone 23GEN, containing LCC2 (SEQ ID NO. 3)

Figure 3 shows the DNA sequence and translation of genomic clone 24GEN, containing LCC3 (SEQ ID NO. 5)

5 Figure 4 shows the DNA sequence and translation of genomic clone 31GEN, containing LCC4 (SEQ ID NO. 7)

Figure 5 shows the DNA sequence and translation of genomic clone 41GEN, containing LCC5 (SEQ ID NO. 9)

Figure 6 shows the structure of vector pMWR1

10 Figure 7 shows the structure of vector pDSY1

Figure 8 shows the structure of vector pDSY10

Figure 9 shows the pH profile of the laccase produced by pDSY2; (A) syringaldazine oxidation; (B) ABTS oxidation.

Figure 10 illustrates a comparison of the use of  
15 laccase vs.  $H_2O_2$ , with various dye precursors, in hair dyeing, as a measurement of DL\*.

Figure 11 illustrates a comparison of the use of laccase vs.  $H_2O_2$ , with various dye precursors, in hair dyeing, as a measurement of Da\*.

20 Figure 12 illustrates a comparison of the use of laccase vs.  $H_2O_2$ , with various dye precursors and modifiers, in hair dyeing, as a measurement of DL\*.

Figure 13 illustrates a comparison of the wash stability of hair dyed with laccase vs.  $H_2O_2$ .

25 Figure 14 illustrates the light fastness of hair dyed with laccase vs.  $H_2O_2$ .

#### Detailed Description of the Invention

*Polyporus pinsitus* is a basidiomycete, also referred to as *Trametes villosa*. *Polyporus* species have previously been  
30 identified as laccase producers (Fahraeus and Lindeberg, *Physiol. Plant.* 6: 150-158, 1953). However, there has been no previous description of a purified laccase from *Polyporus pinsitus*. It has now been determined that *Polyporus*

*pinsitus* produces at least two different laccases, and the genes encoding these laccases can be used to produce relatively large yields of the enzyme in convenient host systems such as *Aspergillus*. In addition, three other genes  
5 which appear to code for laccases have also been isolated.

Initial screenings of a variety of fungal strains indicate that *Polyporus pinisitus* is a laccase producer. The production of laccase by *P. pinsitus* is induced by 2,5-xylidine. Attempts are first initiated to isolate the  
10 laccase from the supernatant of the induced strains. Anion exchange chromatography identifies an approximately 65 kD (on SDS-PAGE) protein which exhibits laccase activity. The enzyme is purified sufficiently to provide several internal peptide sequences, as well as an N-terminal sequence. The  
15 initial sequence information indicates the laccase has significant homology to that of *Coriolus hirsutus*, as well as to an unidentified basidiomycete laccase (Coll et al., Appl. Environ. Microbiol. 59: 4129-4135, 1993. Based on the sequence information, PCR primers are designed and PCR  
20 carried out on cDNA isolated from *P. pinsitus*. A band of the expected size is obtained by PCR, and the isolated fragment linked to a cellulase signal sequence is shown to express an active laccase in *A. oryzae*, but at low levels. One of the PCR fragments is also used as a probe in  
25 screening a *P. pinsitus* cDNA library. In this manner, more than 100 positive clones are identified. The positive clones are characterized and the ends of the longest clones sequenced; none of the clones are found to be full-length.

Further attempts to isolate a full length clone are made.  
30 A 5-6 kb BamHI size-selected *P. pinsitus* genomic library is probed with the most complete cDNA fragment isolated as described above. Initial screening identifies one clone 24GEN(LCC3) having homology to the cDNA, but which is not the cDNA-encoded laccase and also not full length.

Subsequent screening of a 7-8kb BamHI/EcoRI size-selected library indicates the presence of at least two laccases; partial sequencing shows that one, called 21GEN(LCC1), is identical to the original partial cDNA clone isolated, and  
5 the second, called 31GEN(LCC4) is a new, previously unidentified laccase. Secondary screenings of an EMBL4 genomic bank with LCC1 as probe identifies a class of clone containing the entire LCC1 insert as well as the 5' and 3' flanking regions. Screening of the EMBL bank with LCC3  
10 identifies two additional clones encoding laccases which had not previously been identified, 41GEN(LCC5) and 23GEN(LCC2) and which differed structurally from the other three clones LCC1, LCC3, and LCC4. The nucleic acid and predicted amino acid sequences of each of the laccases is presented in  
15 Figures 1-5, and in SEQ ID NOS. 1-10. A comparison of the structural organization of each of the laccases is presented in Table 2. The laccases are generally optimally active at acid pH, between about 4-5.5.

LCC1 is used to create expression vectors, which are in  
20 turn used to transform various species of *Aspergillus*. Transformation is successful in all species tested, although expression levels are highest in *Aspergillus niger*. Shake flask cultures are capable of producing 15 or more mg/liter of laccase, and in lab-scale fermentors, yields of over  
25 300mg/liter are observed. This is a significant improvement over laccase levels observed previously with other laccases and other fungal host cells.

According to the invention, a *Polyporus* gene encoding a laccase can be obtained by methods described above, or any  
30 alternative methods known in the art, using the information provided herein. The gene can be expressed, in active form, using an expression vector. A useful expression vector contains an element that permits stable integration of the vector into the host cell genome or autonomous replication

of the vector in a host cell independent of the genome of the host cell, and preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also include control sequences

5 encoding a promoter, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a signal sequence may be inserted prior to the coding sequence of the gene. For

10 expression under the direction of control sequences, a laccase gene to be used according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can direct the transcription

15 of the laccase gene, include but are not limited to the prokaryotic  $\beta$ -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in

20 "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., Molecular Cloning, 1989.

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be

25 subjected to recombinant DNA procedures, and the choice of vector will typically depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is

30 independent of chromosomal replication, e.g. a plasmid, or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host

cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the laccase DNA sequence should be operably connected to a suitable promoter sequence. The promoter  
5 may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention,  
10 especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*  $\alpha$ -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the  
15 promoters of the *Bacillus amyloliquefaciens*  $\alpha$ -amylase (*amyQ*), or the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes. In a yeast host, a useful promoter is the *eno-1* promoter. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase,  
20 *A. niger* neutral  $\alpha$ -amylase, *A. niger* acid stable  $\alpha$ -amylase, *A. niger* or *A. awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred  
25 are the TAKA-amylase and *glaA* promoters.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to  
30 the DNA sequence encoding the laccase of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter. The vector may further comprise a DNA sequence enabling the vector to

replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

5       The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B.subtilis* or *B.li-*  
*cheniformis*, or one which confers antibiotic resistance such  
as ampicillin, kanamycin, chloramphenicol or tetracycline  
10 resistance. Examples of *Aspergillus* selection markers include *amdS*, *pyrG*, *argB*, *niaD*, *sc*, *trpC* and *hygB*, a marker giving rise to hygromycin resistance. Preferred for use in an *Aspergillus* host cell are the *amdS* and *pyrG* markers of *A. nidulans* or *A. oryzae*. A frequently used mammalian marker is  
15 the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

It is generally preferred that the expression gives  
20 rise to a product which is extracellular. The laccases of the present invention may thus comprise a preregion permitting secretion of the expressed protein into the culture medium. If desirable, this preregion may be native to the laccase of the invention or substituted with a differ-  
25 ent preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase  
30 or proteinase gene from *Rhizomucor miehei*, the gene for the  $\alpha$ -factor from *Saccharomyces cerevisiae* or the calf preprochymosin gene. Particularly preferred, when the host is a fungal cell, is the signal sequence for *A. oryzae* TAKA amylase, *A. niger* neutral amylase, the *Rhizomucor miehei*

aspartic proteinase signal, the *Rhizomucor miehei* lipase signal, the maltogenic amylase from *Bacillus* NCIB 11837, *B. stearothermophilus*  $\alpha$ -amylase, or *B. licheniformis* subtilisin. .

- 5       The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, 10   Sambrook et al. Molecular Cloning, 1989).

- The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the 15   recombinant production of a enzyme of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more 20   likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in 25   connection with the different types of host cells.

- The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus* 30   *licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus* *thuringiensis*, or *Streptomyces lividans* or *Streptomyces*



*murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

- 5       The host cell may also be a eukaryote, such as mammalian cells, insect cells, plant cells or preferably fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of
- 10   *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. Useful filamentous fungi may be selected from a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell.
- 15   Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of
- 20   transforming *Fusarium* species is described by Malardier et al., 1989.

- The present invention thus provides a method of producing a recombinant laccase of the invention, which method comprises cultivating a host cell as described above
- 25   under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase of the
- 30   invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae (e.g. in catalogues of the American Type Culture Collection).

In a preferred embodiment, the recombinant production of laccase in culture is achieved in the presence of an excess amount of copper. Although trace metals added to the culture medium typically contain a small amount of copper, experiments conducted in connection with the present invention show that addition of a copper supplement to the medium can increase the yield of active enzyme many-fold. Preferably, the copper is added to the medium in soluble form, preferably in the form of a soluble copper salt, such as copper chloride, copper sulfate, or copper acetate. The final concentration of copper in the medium should be in the range of from 0.2-2mM, and preferably in the range of from 0.05-0.5mM. This method can be used in enhancing the yield of any recombinantly produced fungal laccase, as well as other copper-containing enzymes, in particular oxidoreductases.

The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like. Preferably, the isolated protein is about 90% pure as determined by SDS-PAGE, purity being most important in food, juice or detergent applications.

In a particularly preferred embodiment, the expression of laccase is achieved in a fungal host cell, such as *Aspergillus*. As described in detail in the following examples, the laccase gene is ligated into a plasmid containing the *Aspergillus oryzae* TAKA  $\alpha$ -amylase promoter, and the *Aspergillus nidulans amdS* selectable marker. Alternatively, the *amdS* may be on a separate plasmid and

used in co-transformation. The plasmid (or plasmids) is used to transform an *Aspergillus* species host cell, such as *A. oryzae* or *A. niger* in accordance with methods described in Yelton et al. (PNAS USA 81: 1470-1474, 1984).

- 5       It is of particular note that the yields of *Polyporus* laccase in the present invention, using *Aspergillus* as host cell are unexpectedly and considerably higher than has previously been reported for expression of other laccases in other host cells. It is expected that the use of
- 10 *Aspergillus* as a host cell in production of laccases from other basidiomycetes, such as *Coriolus* or *Trametes*, will also produce larger quantities of the enzyme than have been previously obtainable. The present invention therefore also encompasses the production of such *Polyporus*-like laccases
- 15 in *Aspergillus* recombinant host cells.

- Those skilled in the art will recognize that the invention is not limited to use of the nucleic acid fragments specifically disclosed herein, for example, in Figures 1-5. It will also be apparent that the invention
- 20 encompasses those nucleotide sequences that encode the same amino acid sequences as depicted in Figure 1-5, but which differ from the specifically depicted nucleotide sequences by virtue of the degeneracy of the genetic code. Also, reference to Figures 1-5 in the specification and the claims
- 25 will be understood to encompass both the genomic sequence depicted therein as well as the corresponding cDNA and RNA sequences, and the phrases "DNA construct" and "nucleic acid sequences" as used herein will be understood to encompass all such variations. "DNA construct" shall generally be
- 30 understood to mean a DNA molecule, either single- or double-stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which are combined and juxtaposed in a manner which would not otherwise exist in nature.

In addition, the invention also encompasses other *Polyporus* laccases, including alternate forms of laccase which may be found in *Polyporus pinsitus* and as well as laccases which may be found in other fungi falling within the definition of *Polyporus* as defined by Fries, or synonyms thereof as stated in Long et al., 1994, ATCC Names of Industrial Fungi, ATCC, Rockville, Maryland. Identification and isolation of laccase genes from sources other than those specifically exemplified herein can be achieved by utilization of the methodology described in the present examples, with publicly available *Polyporus* strains. Alternately, the sequence disclosed herein can be used to design primers and/or probes useful in isolating laccase genes by standard PCR or southern hybridization techniques. Other named *Polyporus* species include, but are not limited to, *P. zonatus*, *P. alveolaris*, *P. arcularius*, *P. australiensis*, *P. badius*, *P. biformis*, *P. brumalis*, *P. ciliatus*, *P. colensoi*, *P. eucalyptorum*, *P. meridionalis*, *P. varius*, *P. palustris*, *P. rhizophilus*, *P. rugulosus*, *P. squamosus*, *P. tuberaster*, and *P. tumulosus*. Also encompassed are laccases which are synonyms, e.g., anamorphs or perfect states of species or strains of the genus *Polyporus*. Strains of *Polyporus* are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), e.g., ATCC 26721, 9385, 11088, 22084, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), e.g., DSM 1021, 1023, and 1182; and Centraalbureau Voor Schimmelcultures (CBS), e.g., CBS 678.70, 166.29, 101.15, 276.31, 307.39, 334.49, and 332.49. The invention also encompasses any variant nucleotide sequence, and the protein encoded thereby, which protein retains at least about an 80% homology, preferably at least about 85%, and most preferably at least about 90-95% homology with any one of the amino acid sequences depicted

in Figures 2-5, and which qualitatively retains the laccase activity of the sequence described herein. Useful variants within the categories defined above include, for example, ones in which conservative amino acid substitutions have  
5 been made, which substitutions do not significantly affect the activity of the protein. By conservative substitution is meant that amino acids of the same class may be substituted by any other of that class. For example, the nonpolar aliphatic residues Ala, Val, Leu, and Ile may be  
10 interchanged, as may be the basic residues Lys and Arg, or the acidic residues Asp and Glu. Similarly, Ser and Thr are conservative substitutions for each other, as are Asn and Gln. It will be apparent to the skilled artisan that such substitutions can be made outside the regions critical to  
15 the function of the molecule and still result in an active enzyme. Retention of the desired activity can readily be determined by conducting a standard ABTS oxidation method, such as is described in the present examples.

The protein can be used in number of different  
20 industrial processes. These processes include polymerization of lignin, both Kraft and lignosulfates, in solution, in order to produce a lignin with a higher molecular weight. Such methods are described in, for example, Jin et al., *Holzforschung* 45(6): 467-468, 1991; US Patent No. 4,432,921;  
25 EP 0 275 544; PCT/DK93/00217, 1992.

The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of  
30 chlorine for depolymerization of lignin, which leads to the production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, Current opinion in

Biotechnology 3: 261-266, 1992; J. Biotechnol. 25: 333-339, 1992; Hiroi et al., Svensk papperstidning 5: 162-166, 1976.

Oxidation of dyes or dye precursors and other chromophoric compounds leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for dye transfer inhibition and dye oxidation can be found in WO 92/01406, WO 92/18683, EP 0495836 and Calvo, Mededelingen van de Faculteit Landbouw-wetenschappen/Rijksuniversitet Gent.56: 1565-1567, 1991; Tsujino et al., J. Soc. Chem.42: 273-282, 1991.

The laccase is particularly well-suited for use in hair dyeing. In such an application, the laccase is contacted with a dye precursor, preferably on the hair, whereby a controlled oxidation of the dye precursor is achieved to convert the precursor to a dye, or pigment producing compound, such as a quinoid compound. The dye precursor is preferably an aromatic compound belonging to one of three major chemical families: the diamines, aminophenols (or aminonaphthols) and the phenols. The dye precursors can be used alone or in combination. At least one of the intermediates in the copolymerization must be an ortho- or para-diamine or aminophenol (primary intermediate). Examples of such are found in Section V, below, and are also described in US Patent No. 3,251,742, the contents of which are incorporated herein by reference. In one embodiment, the starting materials include not only the enzyme and a primary intermediate, but also a modifier (coupler) (or combination of modifiers), which modifier is typically a meta-diamine, meta-aminophenol, or a polyphenol. The modifier then reacts with the primary intermediate in the presence of the laccase, converting it to a colored

compound. In another embodiment, the laccase can be used with the primary intermediate directly, to oxidize it into a colored compound. In all cases, the dyeing process can be conducted with one or more primary intermediates, either  
5 alone or in combination with one or more modifiers. Amounts of components are in accordance with usual commercial amounts for similar components, and proportions of components may be varied accordingly.

The use of this laccase is an improvement over the more  
10 traditional use of  $H_2O_2$ , in that the latter can damage the hair, and its use usually requires a high pH, which is also damaging to the hair. In contrast, the reaction with laccase can be conducted at alkaline, neutral or even acidic pH, and the oxygen needed for oxidation comes from the air,  
15 rather than via harsh chemical oxidation. The result provided by the use of the *Polyporus* laccase is comparable to that achieved with use of  $H_2O_2$ , not only in color development, but also in wash stability and light fastness. An additional commercial advantage is that a single  
20 container package can be made containing both the laccase and the precursor, in an oxygen free atmosphere, which arrangement is not possible with the use of  $H_2O_2$ .

The present laccase can also be used for the polymerization of phenolic or aniline compounds present in  
25 liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such applications have been described in Stutz, Fruit processing  
30 7/93, 248-252, 1993; Maier et al., Dt. Lebensmittel-rindschau 86(5): 137-142, 1990; Dietrich et al., Fluss. Obst 57(2): 67-73, 1990.

Laccases such as the *Polyporus* laccase are also useful in soil detoxification (Nannipieri et al., J. Environ. Qual.

20: 510-517, 1991; Dec and Bollag, Arch. Environ. Contam. Toxicol. 19: 543-550, 1990).

The invention is further illustrated by the following non-limiting examples.

5

#### EXAMPLES

### I. ISOLATION OF A POLYPORUS PINISITUS LACCASE ENZYME

#### MATERIALS AND METHODS

##### 1. Enzymatic assays

Unless otherwise stated, throughout the examples, laccase activity is determined by syringaldazine and 2,2'-bisazino(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), as follows. The oxidation of syringaldazine is monitored at 530 nm with 19  $\mu$ M substrate. In 25 mM sodium acetate, 40  $\mu$ M cupric sulfate, pH 5.5, at 30°C, the activity is expressed as LACU( $\mu$ mole/min). For pH profile studies, Britton & Robinson(B&R) buffers are used, and are prepared according to the protocol described in Quelle, Biochemisches Taschenbuch, H.M. Raven, II. Teil, S.93 u. 102, 1964. ABTS oxidation is carried out with 1mM ABTS in 0.1 M NaAc, pH 5.0 at room temperature by monitoring either  $\Delta$ Abs<sub>405</sub> in a 96-well plate(Costar) or  $\Delta$ Abs<sub>418</sub> in a quartz cuvette. The overlay ABTS oxidase activity assay is carried out by pouring cooled ABTS-agarose(0.03-0.1 g ABTS, 1 g agarose, 50 ml H<sub>2</sub>O, heated to dissolve agarose) over a native IEF gel or PAGE and incubating at room temperature.

##### 2. Initial isolation of laccase

In order to isolate the laccase, 800 ml of culture fluid is filtered by HFSC on a Supra filter(slow filtering). The clear filtrate is then concentrated and washed on an Amicon cell with a GR81 PP membrane to a volume of 72 ml.

One ml aliquots of laccase are bound to a Q-sepharose HP(Pharmacia, Sweden) column, equilibrated with 0.1 M phosphate, pH7 and the laccase is eluted with a NaCl gradient. In all, 10 x 1 ml samples are purified, pooled,



concentrated and washed by ultrafiltration using a membrane with a molecular weight cut-off of 6kD.

### 3. Secondary purification

In a second purification, a fermentation broth is  
5 filtered and concentrated by ultrafiltration. The starting material contains 187 LACU/ml. The concentrate is quick-filtered on a Propex 23 filter(P & S Filtration), with 3% Hyflo Cuper-Cel(HSC; Celite Corporation), followed by two  
10 ultrafiltration on a Filtron filter with two membranes, each with a molecular weight cutoff of 3 kD. The resulting sample (2.5 mS/cm, pH 7.0, at 4°C) is applied to a 130 ml Q-Sephadex column, equilibrated with sodium phosphate, 1.1 mS/cm, pH 7.0. Under these conditions the laccase does not  
15 bind to the column, but elutes slowly from the column during the application and wash with the equilibration buffer, resulting in a partial separation from other brownish material.

This partially purified preparation of 1.0mS, pH 7.0 at 20°C is applied to a Q-sephadex column. The column is  
20 equilibrated with 20mM sodium phosphate, 2.2 mS, pH 7.0. Under these conditions, the laccase binds to the column and is eluted by a gradient of 0-1 M NaCl over 20 column volumes.

### 3. Sequencing

25 For internal peptide sequencing, the purified protein is digested with trypsin, followed by peptide purification with HPLC. Purified peptides are sequenced in an Applied Biosystems 473A sequencer.

## B. RESULTS AND DISCUSSION

### 30 1. Initial characterization

Total yield of the initial purification is about 50 mg(estimated at A280nm). The purified enzyme has a rich blue color, and appears as only two very close bands on SDS-PAGE at about 65 kD. A native PAGE overlaid with substrate

shows that both bands have laccase activity with ABTS. The absorption spectrum shows that besides an absorption at A<sub>280nm</sub>, the purified laccase also shows absorption at about 600nm.

5        2. Sequencing

A N-terminal determination of the protein initially purified shows a single sequence:

Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile-Thr-Asn-Ala-Ala-Ala-Val-Ser-Pro-Asp-Gly-Phe-Pro...

10        Since the N-terminal sequence is not the ideal sequence for constructing a probe, additional experiments with a trypsin digest are conducted, followed by further purification(described above) and sequencing of fragments

2. Secondary purification and characterization

15        In the second purification, the second Q-Sepharose chromatographic step yields the following pools:

Q-Sepharose-2-pool-1 40 ml 112 LACU 47 LACU/A<sub>280</sub>

Q-Sepharose-2-pool-3 80 ml 385 LACU 65 LACU/A<sub>280</sub>

The elution yields >80% of the applied amount. The highly  
20        purified preparation Q-Sepharose-2-pool-3 has an A<sub>280</sub> = 5.9, and A<sub>280</sub>/A<sub>260</sub> = 1.4. The purity of the laccase in the starting material is extremely high on a protein basis but the starting material is a very dark brown color. In SDS-PAGE, a double band is seen, with a dominating 65 kD band  
25        and a smaller 62 kD band. By anionic chromatography, only the dominating band is seen in the first peak(Q-Sepharose-2-pool-1), whereas both bands are seen in the second peak(Q-Sepharose-2-pool-3).

3. Sequence

30        A number of internal peptide sequences are determined, and compared with the *Coriolus hirsutus*(Ch) laccase sequence. The identified fragments are as follows:

Tryp 13:

Ser-Pro-Ser-Thr-Thr-Thr-Ala-Ala-Asp-Leu

Tryp 14:

Ser-Ala-Gly-Ser-Thr-Val-Tyr-Asn-Tyr-Asp-Asn-Pro-Ile-Phe Arg

Tryp 16:

Sequence 1:

5 Ser-Thr-Ser-Ile-His-Trp-His-Gly-Phe-Phe-Gln-Lys

Sequence 2:

Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile-Thr-Asn-Ala-Ala-Val

Tryp 18:

Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile-Thr-Asn

10 Tryp 19:

Sequence 1:

Leu-Gly-Pro-Ala-Phe-Pro-Leu-Gly-Ala-Asp-Ala-Thr-Leu-Ile-

Sequence 2:

Phe-Gln-Leu-Asn-Val-Ile-Asp-Asn-Asn-Thr-Thr-His-Thr-Met

15 Tryp 25:

Tyr-Ser-Phe-Val-Leu-Glu-Ala-Asn-Gln-Ala-Val-Asp-Asn-Tyr-Trp-Ile-Arg

Tryp 27

Gly-Thr-Asn-Trp-Ala-Asp-Gly-Pro-Ala-Phe

20 II. ISOLATION OF A POLYPORUS PINISITUS LACCASE CDNA CLONE

A. MATERIALS AND METHODS

1. RNA preparation

RNA is isolated from 10 grams of *P. pinsitus* mycelium grown under xyloidine induction for 6.5 hours, using the  
25 guanidium/CsCl cushion method. The RNA is poly-A selected on an oligo-dT column, using standard conditions. 120µg mRNA is obtained and stored as lyophilized pellet in 5µg aliquots at -80°C.

2. Single stranded cDNA

30 Single stranded cDNA is synthesized using the reverse transcriptase "Super Script" (BRL) according to manufacturer's directions.

3. Construction of cDNA library

A cDNA library is constructed using the librarian IV cDNA kit (Invitrogen). Fifty cDNA pools, each containing approximately 5000 individual transformants, are obtained.

#### 4. PCR

- 5 PCR is conducted under the following standard conditions: 100pmol of each primer, 10 $\mu$ l 10X PCR buffer(Perkin-Elmer), 40 $\mu$ l dNTP 0.5 mM, 2 $\mu$ l single stranded cDNA(or approximately 100 ng chromosomal DNA or 100 ng PCR fragment), H<sub>2</sub>O to 100  $\mu$ l, 2.5U Taq polymerase. The cycles  
10 are 3x(40°C/two minutes, 72°C/two minutes, 94°C/one minute) followed by 30x(60°C/two minutes, 72°C/two minutes, 94°C/1 minute).

### B. RESULTS AND DISCUSSION

#### 1. Cloning of *Polyporus pinsitus* laccase

- 15 PCR is carried out with the primer #3331:  
ACCAGNCTAGACACGGGNTC/AGATACTG/ACGNGAGAGCGGAC/TTGCTGGTC  
ACTATCTTCGAAGATCTCG  
and primer #3332:  
CGCGGCCGCTAGGATCCTCACAATGGCCAA/CTCTCTG/CCTCG/ACCTTC.  
20 A clear band of about 1500bp is obtained. The DNA is digested with NotI/HindIII, and fractionated on an agarose gel. The upper band(fragment #42) is purified and cloned into the *Aspergillus* vector pHD423. No transformants are obtained. Several attempts are carried out in order to  
25 clone the fragment, including redigestion with the restriction enzymes, phosphorylation of the ends, filling in with klenow and blunt-end cloning in SmaI cut pUC18, without success. Hybridization with a laccase probe based on the laccase described in Coll et al., *supra*, indicates that the  
30 PCR product could be the *P. pinsitus* laccase. In a new attempt to clone the PCR fragment, a new PCR reaction is carried out, using the same conditions as for fragment #42. Again the result is a fragment of about 1500 bp(fragment #43). This time the fragment is cut with HindIII/BamHI, and

ligated to HindIII/BamHI-cut pUC18. Three clones, #43-/A,-B,-G are found to contain a fragment of 1500 bp. Partial sequencing reveals that these fragments are laccase related.

#### 2. Expression of *Polyporus pinsitus* laccase

5 To express the laccase, the fragment #43 is joined to a signal sequence from a 43kD cellulase. The primer pHD433 (TAGCGGATCCCACAATGCGTTCCTCCCCCTCCTCCGTCGCGCGTTGTGGCCGCCCTG CCGGTGTTGGCCCTTGCCGGCATTGGGCGCGTCGCGGACC) is used in a standard PCR reaction with a pUC forward primer (New England  
10 Biolabs). All three clones are used as templates in order to minimize the risk of working with DNA containing errors.

The PCR generated DNA from the reaction with a primer pHD433 and template 43-A and 43-G is cut with HindIII/BamHI and cloned into the *Aspergillus* expression vector  
15 pHD414 (described in detail below). Several transformants are obtained.

Clones pHD433/43A-1,2, pHD433/43G-2,-3 are transformed into *A. oryzae*. The transformants from each transformation (between 3-10) are analyzed for laccase production.

20 Activity is only obtained with pHD433/43G-3. The positive transformants (numbers 1, 4, 6) are reisolated on amdS plates, and retested. In an additional transformation round a further ten transformants are obtained with pHD433/43G-3. The clones #20, 23, 26, 28, and 29 are positive. The clones  
25 are reisolated and two single isolates are analyzed for laccase expression semiquantitatively by color development in an ABTS assay at pH 4.5. On a scale of +-+++, several clones show moderate to strong expression of laccase.

Further cloning is conducted to identify a full length  
30 clone. A xyloidine-induced cDNA library consisting of approximately 350,000 transformants is screened using fragment #42-4 as a probe. More than 100 positive clones are detected. The clones are purified, rescreened, and analyzed on Southern blots. Two of the longest clones are

further characterized by DNA sequence determination. The longest clones are found to be identical and found to contain a poly-A stretch in the 3' end and to start at the amino acid number 4 in the amino terminus. A partial DNA  
5 sequence is determined from different clones.

phD433/43G-3 is then used in further cloning studies as described in the following Section IV.

### III. PURIFICATION AND CHARACTERIZATION OF ADDITIONAL POLYPORUS PINSITUS LACCASE WILD-TYPE ENZYMES

#### 10 A. MATERIALS AND METHODS

##### 1. Culture conditions

Shake flasks (250 ml medium/2.8 l baffled flask) are inoculated with several agar plugs taken from a week-old PDA plate of *P. pinsitus*. The medium contains, per liter, 10 g  
15 glucose, 2.5 g L-asparagine, 0.2 g L-phenylalanine, 2.0 g yeast extract, 2.0 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0 ml AMG trace metals, 0.002 g  $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0 g citric acid, made with tap water, pH 5.0 before autoclaving. The cultures are grown at 18-22°C on a rotary shaker with low agitation (~100 rpm).  
20 After 7 days, the pH of each shake flask is adjusted to ~6.0 by the addition of 0.25 ml 5 N NaOH and the cultures are induced by adding 0.5 ml of a 2,5-xylidine stock solution (xylidine diluted 1:10 into ethanol) to each flask. Flasks are incubated for an additional 24 hours, at which  
25 time the culture supernatant from each flask is recovered.

##### 2. Materials.

Chemicals used as buffers are commercial products of at least reagent grade. Endo/N-glucosidase F is from Boehringer-Mannheim. Chromatography is performed on  
30 Pharmacia FPLC. Spectroscopic assays are conducted on either a spectrophotometer (Shimadzu PC160) or a microplate reader (Molecular Devices).

##### 3. Purification

Culture broth is filtered first on cheesecloth and centrifuged at 1000 x g to remove gelatinous pinkish xylidine polymer. The supernatant is then filtered on Whatman #2 paper and concentrated from 1500 to 250 ml on  
5 SLY100 (Amicon, Spiral concentrator) at 4°C. The concentrated broth is diluted with water until it reaches 0.8 mS (from 2.5 mS) and then concentrated on SLY100 to 250 ml. The washed broth, thawed from -20°C freezing overnight, is subjected to Whatman #2 paper filtration to remove  
10 residual pinkish material, and then pH adjusted by NaOH from pH 6.1 to pH 7.7. This yellowish broth, 275 ml with 0.8 mS, is applied on a Q-Sepharose XK-26 column (~64 ml gel) equilibrated with 10 mM Tris-HCl, pH 7.7, 0.7 mS. The first active laccase fraction runs through during loading and  
15 washing by the equilibrating buffer. The elution is carried out by a linear gradient of 0-0.5 M NaCl in the equilibrating buffer over 8.8 bed-volume. The second and third active fractions are eluted around 0.15 and 0.35M NaCl, respectively. No more active fractions are detected.  
20 when the column is washed sequentially with 2 M NaCl and with 1 mM NaOH. The active fractions are pooled, adjusted to ~10mS, concentrated on Centricon-10 (Amicon), and then applied onto Superdex 75 (HR10/30, 24 ml, Pharmacia) equilibrated with 10mM Tris-HCl, 0.15 M NaCl, pH 8, 14 mS.  
25 During elution with the application buffer, laccase fractions are eluted off using the same elution volume for all three Q-Sepharose fractions, indicating very similar native molecular weight. The purity of the laccase is tested on SDS-PAGE.

#### 30 4. Protein analysis

PAGE and native IEF are carried out on a Mini Protean II and a Model 111 Mini IEF cells (Bio-Rad). Western blots are carried out on a Mini trans-blot cell (Bio-Rad) with an alkaline phosphatase assay kit (Bio-Rad). The primary

antibodies are diluted 1000-fold during blotting. N-terminus sequencing is performed on an Applied Biosystems (ABI) 476A protein sequencer using liquid phase TFA delivery for cleavage and on-line HPLC for identification of PTH-  
5 amino acids. Standard Fast Cycles and Pre-Mix Buffer System is used according to manufacturer's instructions. Deglycosylation with glycosidase is done as follows: 3µg of protein and 3.6 units of glycosidase in 0.25M NaAc, pH 5, 20 mM EDTA, 0.05% 2-mercaptoethanol is incubated at 37°C for 18  
10 hours with ovalbumin and bovine serum albumin serving as positive and negative control, respectively, and the mobility is detected by SDS-PAGE.

Amino acid analysis for determining extinction coefficients is done using Amino Quant 1090 HPLC system from  
15 Hewlett Packard. Microwave facilitated vapor phase hydrolysis of lyophilized samples is done using the MDS-2000 hydrolysis-station(CEM, Matthews, NC). 6N HCl containing 1% phenol as a scavenger is used to create the acid vapors. Hydrolysis time is 20 minutes at 70 psi (~148°C).  
20 Hydrolyzed samples are lyophilized and redissolved in 20 µl of 500pmol/µl sarcosine and norvaline as internal standards. 1µl is injected and analyzed according to manufacturer's instructions.

## B. RESULTS AND DISCUSSION

### 25 1. Purification

The previously characterized *P. pinsitus* laccase has a pI of ~3.5. However, considerable laccase activity is detected in the run-through fraction of Q-Sepharose pre-equilibrated at pH 7.7. Upon a gradient elution, one more  
30 active fraction comes off the column before the active fraction initially anticipated. UV-visible spectra and SDS-PAGE show that all three fractions contain mainly laccase. After further purification by gel filtration, different pI's under native non-denaturing conditions are detected for the



two new fractions and shown to be consistent with the elution order.

## 2. Characterization

The pure laccase preparations derived from Q-Sepharose eluates behave as a rather well-defined band on SDS-PAGE at ~63 kDa. Deglycosylation detects ~14% w/w carbohydrates based on mobility change on SDS-PAGE. On native-IEF, the laccase preparations have bands of pI 6-6.5, 5-6.5, and 3.5. ABTS-agarose overlay show that all bands are active. Each form in turn shows multiple isoforms under the IEF conditions.

The neutral and acidic forms have a typical UV-visible spectrum with maxima at 605 and 275 nm. The ratio of  $A_{275}/A_{605}$  is 30-40. The spectrum for the acidic-neutral form has a peak at 276 nm and a shoulder around 600 nm.

The N-terminal sequencing shows that the neutral and neutral-acidic forms have the same first 29 residues (Table 1). The N-terminus of the acidic form matches 100% to that of the previously characterized form. All three forms exhibit comparable cross-reactivity toward antibodies raised against previously characterized form.

Table 1. Structural and enzymatic properties of *P. pinsitus* laccases

	<u>Form</u>	<u>N-terminus</u>	<u>LACU</u>	<u><math>\Delta A_{405\text{min}}^{-1}(\text{ABTS})</math></u>
5	Acidic	GIGPVA D LTITNAAVSPDGFSRQAVVNG	92	4000
	Acidic-	A*****(*)*VVA**P*****L*D*I*****	75	4000
	Neutral			
	Neutral	A*****(*)*VVA**P*****L*D*I*****	32	1000

10 \*:Same residue as compared with the acidic form. (): weak signal

### 3. Laccase Activity

The specific activities(per  $A_{275}$ ) of the three forms are tested by both ABTS and syringaldazine oxidations. The shapes and optima of the pH activity profiles for the three forms are very close: all have optima at  $\leq \text{pH}4$  and pH 5-5.5 for ABTS and syringaldazine oxidations, respectively.

## IV. ISOLATION OF MULTIPLE COPIES OF POLYPORUS PINSITUS

### 20 LACCASE ENZYMES AND GENES

#### A. MATERIALS AND METHODS

##### 1. Strains

The following strains are employed in the methods described below: *E. coli* K802(e14-(mrca), mcrB, hsdR2, galk2, galT22, supE44, metB1; Clonetech); *E. coli* XL-1 Blue(recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F'proAB, lacIqZDM15, Tn10(tet<sup>r</sup>)] ;Stratagene) and *Polyporus pinsitus* CBS 678.70.

##### 2. Genomic DNA isolation

30 Cultures of *P.pinsitus* are grown in 500 ml YG (0.5% yeast extract, 2% dextrose) at room temperature for 3 to 4 days. Mycelia are harvested through miracloth, washed twice with TE and frozen quickly in liquid nitrogen. The frozen mycelia are stored at -80°C. To isolate DNA, the mycelia

are ground to a fine powder in an electric coffee grinder. The powdered mycelia are resuspended in TE to a final volume of 22 ml. Four ml 20% SDS is added with mixing by inversion followed by incubation at room temperature for 10 minutes.

5 The sample is gently extracted with phenol:chloroform and centrifuged to separate the phases. The aqueous phase is collected and 400µl proteinase A(10 mg/ml stock) is added. The sample is incubated at 37°C for 30 minutes followed by a phenol:chloroform extraction. The aqueous phase is

10 precipitated by the addition of 0.1 volumes of 3 M Na acetate, pH 5.2 and 2.5 volumes 95% ethanol and freezing at 20°C for one hour. After centrifugation to precipitate the DNA, the pellet is resuspended in 6 ml TE, and 200 µl boiled RNase A(10 mg.ml stock) is added. After incubation at 37°C,

15 100 µl proteinase A(10 mg/ml stock) is added followed by incubation at 37°C for 30 minutes. The sample is phenol:chloroform extracted twice. To the aqueous phase, 0.1 volumes 3 M Na acetate and 2.5 volumes are added, and the sample is frozen at -20°C for 1 hour. Following

20 centrifugation, the pellet is gently resuspended in 400 µl TE, and 40 µl Na acetate and 1 ml 95% ethanol are added. The DNA is pelleted by centrifugation, and the pellet is washed in 70% ethanol. The final pellet is resuspended in 250 µl TE.

25        3. RNA preparation

RNA is isolated from mycelia which are harvested from *P. pinisitus* cultures which are either induced for laccase expression by the addition of 2,5-xygidine or are uninduced. The mycelia are washed and frozen quickly in liquid N<sub>2</sub>.

30 Frozen mycelia are ground to a fine powder in an electric coffee grinder. The powder is immediately suspended in 20 ml extraction buffer (0.2 M Tris-HCl, 0.25 M NaCl, 50 mM EGTA, 0.8% tri-isopropyl naphthalene sulfonic acids, 4.8% p-aminosalicylic acid, pH 8.5). All solutions for RNA

extraction are made with diethylpyrocarbonate (DEP)-treated water. The sample is kept on ice and 0.5 volumes TE-saturated phenol:chloroform is added. The sample is mixed well by inversion for 2 minutes, and the phases are  
5 separated by centrifugation. The aqueous phase is saved, and the organic phase is extracted with 2 ml extraction buffer and incubated at 68°C for 5 minutes. After centrifugation to separate the phases, the aqueous phases are pooled and extracted several time with phenol:chloroform  
10 until there is no longer any protein at the interface. To the aqueous phase 0.1 volume 3 M Na-acetate, pH 5.2 and 2.5 volumes 95% ethanol are added to precipitate the RNA, and the sample is frozen at -20°C for 2 hours. The RNA is pelleted and resuspended in DEP water with RNase inhibitor.

15 4. DNA sequencing

Nucleotide sequences are determined using TAQ polymerase cycle sequencing with fluorescent-labeled nucleotides, and reactions are electrophoresed on an Applied Biosystems automatic DNA sequencer (Model 363A, version  
20 1.2.0).

5. Preparation of genomic libraries

Two size-selected genomic libraries of *P. pinsitus* are constructed. A library of 5 to 6 kb BamHI fragments are constructed in pBluescript+. Genomic DNA is digested with  
25 BamHI, and the digest is electrophoresed on a preparative agarose (IBI) gel. The region containing the 5 to 6 BamHI fragments is sliced from the gel. The DNA is isolated from the gel using a GeneClean kit (BIO 101). The DNA is ligated into pBluescript plasmid previously digested with BamHI and  
30 dephosphorylated with BAP (GIBCO BRL), *E. coli* XL-1 Blue competent cells (Stratagene) are transformed with the ligation, and 12,000 white colonies are obtained.

A library of 7 to 8 kb BamHI/EcoRI fragments is constructed in pUC118. Ten µg genomic DNA is digested with

BamHI and EcoRI and treated with BAP(GIBCO BRL). Competent *E. coli* XL-1 Blue cells are transformed with the ligation, and the library contains ~8000 recombinants.

For the preparation of a total genomic library in  
5 lambda EMBL4, 25 µg of *P. pinsitus* genomic DNA is partially digested with Sau3A. After digestion, the DNA is electrophoresed on a preparative low-melt agarose gel, and a band containing the 9 to 23 kb sized DNA is sliced from the gel. The DNA is extracted from the gel using β-agarose(New  
10 England Biolabs). The isolated EMBL4 arms (Clonetech) according to the supplier's directions. The ligation is packaged *in vitro* using a Gigapack II kit(Stratagene). The library is titered using *E. coli* K802 cells. The unamplified library is estimated to contain 35,000  
15 independent recombinants. The library is amplified using *E. coli* K802 cells.

#### 6. Southern and Northern Blots

DNA samples are electrophoresed on agarose gels in TAE buffer using standard protocols. RNA samples are  
20 electrophoresed on agarose gels containing formaldehyde. Both DNA and RNA gels are transferred to Zeta-Probe membrane(BIO-RAD) using either capillary action under alkaline conditions or a vacuum blotter. After transfer, the DNA gels are UV crosslinked. Blots are prehybridized at  
25 65°C in 1.5X SSPE, 1% SDS, 0.5% non-fat dried milk and 200 µg/ml salmon sperm DNA for 1 hour. Radioactive probes are added directly to the prehybridization solutions, and hybridizations are continued overnight at 65°C. Blots are washed with 2XSSC for 5 minutes at 65°C and with 0.2XSSC,  
30 1%SDS,0.1% Na-pyrophosphate at 65°C for 30 minutes twice.

Radioactive labeled probes are prepared using a α-<sup>32</sup>P-dCTP and a nick translation kit(GIBCO-BRL).

#### 7. Library screening

For screening of the size-selected 5-6 kb BamHI and 7-8 kb BamHI/EcoRI libraries -500 colonies on LB carb plates and lifted the colonies to Hybond N<sup>+</sup> filters(Amersham) using standard procedures. The filters are UV crosslinked following neutralization. The filters are prehybridized at 65°C in 1.5X SSPE, 1% SDS, 0.5% non-fat dried milk, 200 µg/ml salmon sperm DNA for 1 hour. Nick-translated probes are added directly to the prehybridization solution, and hybridizations are done overnight at 65°C.

- 10 For screening of the genomic bank in EMBL, appropriate dilutions of the amplified library are plated with *E. coli* K802 cells on 100mM NZY top agarose. The plaques are lifted to Hybond N<sup>+</sup> membranes(Amersham) using standard procedures. The DNA is crosslinked to the membranes using UV crosslinking. The filters are prehybridized and hybridized using the same conditions as those mentioned above.

#### RESULTS AND DISCUSSION

##### 1. Isolation of multiple copies of laccase gene

- P. pinsitus* genomic DNA is digested with several different restriction enzymes for southern analysis. The blot is probed with the cDNA insert(isolated as a BamHI/SphI fragment from the pYES vector) which is labeled with α-P<sup>32</sup>-dCTP. The blot is hybridized and washed as described above. The cDNA hybridizes to several restriction fragments for most of the enzymes suggesting that there are multiple laccase genes in the genome. Because the cDNA hybridizes to a BamHI fragment of ~5.5 kb, a library of 5-6 kb BamHI fragments from *P. pinisitus* is constructed.

##### 2. Screening of Genomic Libraries

- 30 The results from screening of the libraries are summarized in Table 2. The 5-6 kb BamHI size-selected library is screened with the original cDNA clone labeled with <sup>32</sup>P. Approximately 30,000 colonies are screened with hybridizations done at 65°C. Plasmid DNA is isolated from

two positive colonies and digested with BamHI to check for insert size. Both clones contain an ~5.5 kb BamHI insert. The cloned insert(LCC3) is sequenced from either end; the sequence has homology to the cDNA, but is clearly not the  
5 cDNA encoded laccase. The partial DNA sequence of LCC3 also indicates that the LCC3 pUC118 clone does not contain the full gene.

From a southern blot of BamHI/EcoRI double digested DNA it is demonstrated that the cDNA hybridizes to an ~7.7 kb  
10 fragment. A size-selected library in pUC118 is constructed containing 7-8 BamHI/EcoRI fragments. A total of ~8000 independent colonies are obtained and screened by hybridization with a <sup>32</sup>P labeled insert. Plasmid DNA is isolated from the positive colonies and digested with BamHI  
15 and EcoRI. Restriction analysis of the plasmids demonstrate that they fall into two classes. One class (LCC4) contains four clones which are all identical and have an ~7.7 kb BamHI/EcoRI insert which hybridizes to the cDNA. A second class(LCC1) contains two clones which are identical and have  
20 inserts of ~7.2 kb which hybridize to the cDNA. Partial DNA sequencing of clones LCC1 and LCC4 demonstrate that clone 21 is the genomic clone of the original cDNA, while LCC4 codes for another laccase. The partial DNA sequence of LCC1 shows that the pUC118 clone does not contain the full gene and  
25 that a fragment upstream of the EcoRI site is needed.

At the same time the size selected 7-8 BamHI/EcoRI library is being constructed, a *P. pinisitus* genomic bank in EMBL4 is constructed containing ~35,000 independent recombinant phage. Ten positive plaques are picked and  
30 purified. DNA is isolated from the purified phage lysates. Restriction digests of EMBL DNAs demonstrates that there are three classes of clones. The first class(11GEN) is defined by two sibs whose inserts contain a BamHI/EcoRI fragment of the same size as LCC1 which hybridizes to the LCC1 insert.

The second class(12GEN) contains one clone which has a different restriction pattern than the 11GEN class and whose insert contains a different restriction pattern than the 11GEN class and whose insert contains an ~5.7 kb BamHI/EcoRI fragment. The third class is defined by a single clone whose insert contains an ~3.2 kb BamHI/EcoRI fragment which hybridizes to the LCC1 insert. DNA sequence analysis demonstrates that clone 11GEN contains the LCC1 BamHI/EcoRI fragment and both 5' and 3' flanking regions. It is also demonstrated that clone 12GEN contains a portion of the LCC1 insert.

The *P. pinisitus* EMBL genomic bank is also screened with the LCC3 BamHI insert in order to clone the full gene. Approximately 30,000 plaques are plated and lifted from hybridization. Five plaques which hybridize to the LCC3(BamHI/EcoRI) insert are identified and purified. DNA is isolated from the purified phage stocks. Southern analysis of *P. pinisitus* genomic DNA demonstrates that the LCC3 BamHI insert hybridizes to an ~7kb EcoRI fragment. Restriction digests and southern demonstrate that 4 of the clones contain restriction fragments which hybridize to the EcoRI/BamHI(1.6 kb) fragment and that the clones fall into three classes. Class one is defined by a single clone(LCC5) whose insert contains a 3kb EcoRI fragment which hybridizes to the LCC3 BamHI/EcoRI fragment. Another class is defined by clone(LCC2) whose insert contains an ~11 kb EcoRI fragment which hybridizes to the LCC3 BamHI/EcoRI insert. The third class is defined by two clones which are not identical but contain many of the same restriction fragments; these clones both contain an ~7.5 kb EcoRI fragment which hybridizes to the LCC3 insert. Further analysis of this third class indicates that they are identical to clone LCC4. Partial DNA sequencing of LCC5 and LCC2 indicates that both of these clones code for laccases;



however, neither is identical to any of the above mentioned laccase genes (LCC1, LCC3, or LCC4). At this point, five unique laccase genes are cloned; however, the fragments subcloned from LCC5 and LCC2 do not contain the full genes.

5 From the DNA sequencing of the 3 kb EcoRI fragment from clone LCC5 it is determined that ~200 base pairs of the N-terminus are upstream of the EcoRI site. A 380 bp EcoRI/MluI fragment from LCC5 is used to identify for subcloning a MluI fragment from the LCC5 EMBL clone. An  
10 ~4.5 MluI fragment from the LCC5 EMBL clone is subcloned for sequencing and shown to contain the N-terminal sequence.

To clone the N-terminal half of the LCC3 laccase gene, the *P. pinsitus* EMBL genomic bank is probed with an ~750 bp BamHI/StuI restriction fragment from the LCC3 pUC118 clone.  
15 Approximately 25,000 plaques are screened and five plaques appear to hybridize with the probe. Upon further purification only three of the clones are still positive. Two of the clones give very strong signals and the restrictions digests of DNA isolated from these phage  
20 demonstrate that both contain an ~750 bp BamHI/StuI fragment in their inserts and that the two clones are not identical but overlapped. Based on results of Southern analysis, an ~8.5 kb fragment from these clones are subcloned for sequencing. The EcoRI fragment is shown to contain the  
25 entire gene.

To clone the N-terminal half of the LCC2 laccase gene, the *P. pinsitus* genomic bank in EMBL4 is probed with an ~680 bp EcoRI/PvuI of the EMBL LCC2 clone. Thirty thousand plaques are screened by hybridization at 65°C, and 15  
30 plaques appear to hybridize with the probe. All fifteen are purified, and DNA is isolated. The clones can be placed in four classes based on restriction patterns. Seven of the clones are all sibs, and are identical to the original EMBL clone of LCC2. The second class is defined by 3 clones

which are sibs. An ~4 kb HindIII fragment is subcloned from this class for sequencing and is shown to contain the N-terminal half of LCC2. A third class is defined by a single clone and is not characterized further.

5        3. DNA sequencing

The complete DNA sequences of the five genomic clones is determined as described in Materials and Methods. Sequencing of clone LCC2 demonstrate that it probably codes for the second form of laccase(neutral pI) isolated from  
10 culture broth from an induced *P. pinsitus* culture as described above. The N-terminal protein sequence from the neutral pI laccase and the predicted N-terminus for the protein coded for by LCC2 are compared, and show identity. The predicted pI for the protein coded for by clone LCC2 is  
15 5.95, which is in good agreement with the experimental pI determined for the second form of laccase being between 5.0 and 6.5. Figures 1-5 (SEQ ID NOS. 1-5) show the DNA sequences and predicted translation products for the genomic clones. For LCC1, the N-terminus of the mature protein as  
20 determined by protein sequencing and predicted by Von Heijne rules is Gly at position 22. The N-terminus is Gly-Ile-Gly-Pro-Val-Ala-. For LCC2 the N-terminal amino acid of the mature protein as determined by protein sequencing is Ala at position 21. The N-terminus is Ala-Ile-Gly-Pro-Val-Ala-.  
25 For LCC3 the predicted N-terminal amino acid of the mature protein is Ser at position 22, with the N terminus being Ser-Ile-Gly-Pro-Val-Thr-Glu-Leu-. For LCC4, the predicted N-terminal amino acid is Ala at position 23 with the N-terminus being Ala-Ile-Gly-Pro-Val-Thr-. For LCC5 the  
30 predicted N-terminal amino acid is Ala at position 24 with the N-terminus being Ala-Ile-Gly-Pro-Val-Thr-Asp. A comparison of the structural organization of the genes and the predicted proteins they code for is presented in Table 1. It will be seen that the five genes have different

structural organizations and code for proteins of slightly different sizes. Comparisons between the predicted proteins of the genomic clones and other fungal laccase are also done. Table 2 shows a comparison of the predicted laccase to each other and to other fungal laccases. Clone LCC1 (the induced laccase first characterized) has the most identity (90%) to the *Coriolus hirsutus* laccase and the PM1 basidiomycete laccase (Coll et al., *supra*). The other four laccases have between 64 and 80% identity to the *C. hirsutus* laccase. The laccase coded for by LCC3 has the least identity to the LCC1 laccase and the other fungal laccases shown in Table 2. LCC2 appears to be the second wild-type laccase isolated as described above; based on the N-terminal sequences of the isolated clones, it also appears that the "neutral" and acidic neutral" wild-type laccases are the same enzyme which is encoded by the LCC2 sequence.

Table 1 Comparison of Structural Organization and Predicted Proteins of the *P. pinisiis* Genomic Clones.

<u>Gene</u>	<u># Introns</u>	<u>Size of Predicted Precursor Protein</u>	<u>Size of Predicted Mature Protein</u>	<u>Predicted Isoelectric Point</u>
21GEN	8	520	499	4.49
23GEN	10	519	498	5.95
24GEN	12	516	495	5.23
31GEN	11	510	488	4.06
41GEN	11	527	504	4.07

Table 2 Amino Acid Identity Between *P. pinsitis* Laccases and Other Fungal Laccases.

	21GEN	23GEN	24GEN	31GEN	41GEN	CRIPHA	CRIPHE	PBILAC	PM1
21GEN	_____	79%	64%	70%	72%	90%	91%	64%	80%
23GEN	79%	_____	65%	66%	69%	80%	81%	62%	74%
24GEN	64%	65%	_____	61%	65%	64%	65%	61%	63%
31GEN	70%	66%	61%	_____	75%	69%	70%	64%	69%
41GEN	72%	69%	65%	75%	_____	71%	72%	64%	71%
CRIPHA	90%	80%	64%	69%	71%	_____	99%	64%	80%
CRIPHE	91%	81%	65%	70%	72%	99%	_____	65%	81%
PBILAC	64%	62%	61%	64%	64%	64%	65%	_____	65%
PM1	80%	74%	63%	69%	71%	80%	81%	65%	_____

21GEN, 23GEN, 24GEN, 31GEN and 41GEN= *P. pinsitis* laccase clones

CRIPHA= *Coriolus hirsutis* laccase A

CRIPHE= *C. hirsutis* laccase B

PBILAC= *Phlebia radiata* laccase

PM1= Basidiomycete PM1 laccase (CECT2971)

### 5. Northern blots

RNA is isolated from mycelia from both a xyloidine-induced culture and an uninduced culture. RNA is blotted to membrane after electrophoresis, and the blot is probed with the cDNA insert, or a small fragment containing ~100 bp of the 23GEN promoter and the first 100 bp of the coding region. A transcript of about 1.8 kb hybridizes to both the induced and uninduced RNA samples; however, transcription of this message is clearly induced by the addition of xyloidine to the culture.

## III. EXPRESSION OF *P. PINSITUS* LACCASE IN *ASPERGILLUS*

### MATERIALS AND METHODS

#### 1. Strains

*A. oryzae* A1560, *A. oryzae* HowB104 (fungamyl delete, pyrg), *A. oryzae* HowB101pyrg, *A. niger* Bo-1, *A. niger* Bo-80, *A. niger* ATCC1040, *A. niger* NRRL337, *A. niger* NRRL326, *A. niger* NRRL326, *A. niger* NRRL2295, *A. niger* ATCC11358, *A. niger* NRRL322, *A. niger* AT10864, *A. japonicus* A1438, *A. phoenicis*, *A. foetidus* N953.

#### 2. Media

For the shake flask cultivation of the *A. niger*, *A. foetidus*, and *A. phoenicis* MY50 (per liter: 50 g maltodextrin, 2 g  $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ , 10 g  $\text{KH}_2\text{PO}_4$ , 2 g  $\text{K}_2\text{SO}_4$ , 2 g citric acid, 10 g yeast extract, 0.5 ml trace metals, 2 g urea, pH 6.0) media is used. For the shake flask cultivation of the *A. oryzae* A1560 and HowB101 strains MY51 (per liter: 30 g maltodextrin, 2 mg  $\text{MgSO}_4$ , 10 g  $\text{KH}_2\text{PO}_4$ , 2 g  $\text{K}_2\text{SO}_4$ , 2 g citric acid, 10 g yeast extract, 0.5 ml trace metals, 1 g urea, 2 g  $(\text{NH}_4)_2\text{SO}_4$ , pH 6.0) is used. For the shake flask analysis of the *A. oryzae* HowB104 strains, MY51 maltose (same as MY51 but with 50g of maltose instead of maltodextrin) media is used. For the shake flask analysis of the *A. japonicus* strains M400 media (per liter: 50 g maltodextrin, 2 g  $\text{MgSO}_4$ , 2 g

KH<sub>2</sub>PO<sub>4</sub>, 4 g citric acid, 8 g yeast extract, 0.5 ml trace metals, 2 g urea, pH 6.0.

Cultures grown overnight for protoplast formation and subsequent transformation are grown in YEG(0.5% yeast extract, 2% dextrose). For strains that are *pyrg*, uridine is supplemented to 10 mM final concentration.

### 3. Screening for laccase production

Primary transformants are screened first on a minimal medium plates containing 1% glucose as the carbon source and 1mM ABTS to test for production of laccase. Transformants that give green zones on the plates are picked and spore purified before shake flask analysis is done.

Shake flask samples are centrifuged to clear the broth. Dilute or undiluted broth samples are assayed with ABTS

15

## RESULTS AND DISCUSSION

### 1. Expression in shake flasks

The first expression vector constructed is pDSY1, which contains the TAKA promoter, TAKA signal sequence, P.

*pinisitus* laccase cDNA beginning at the mature N-terminus and the AMG terminator. The TAKA signal sequence: laccase insert is constructed in 2 steps. First by site directed mutagenesis, an AgeI site beginning at bp 107 of the laccase mature coding region is created by a single base change and a NsiI site is created ~120 bp downstream of the laccase stop codon(ACG GGT->ACC GGT and TTC GCT->ATG CAT, respectively). A small PCR fragment beginning with an SfiI site and ending with the AgeI site at 107 bp in laccase is PCR amplified. This fragment contains a piece of the TAKA signal sequence and the first ~107 bp of the mature laccase cDNA. Further DNA sequencing of this fragment shows it has a single base change that leads to a substitution of Asn for Thr at position 9 in mature laccase. This substitution creates a potential N-linked glycosylation site. The PCR

fragment and AgeI/NsiI fragments are cloned into pMWR1 (Figure 6) which has been digested with SfiI/NsiI. The vector pMWR1 contains the TAKA promoter, a portion of the TAKA signal sequence which ends with an SfiI site, and the  
5 TAKA terminator with a NsiI site inserted directly 5' to the terminator. The resulting expression vector (Figure 7) is used to cotransform several hosts. Methods for co-transformation of *Aspergillus* strains are as described in Christensen et al., *supra*.

- 10 In the second laccase expression vector, the base change in DSY1 which leads to the substitution of Asn for Thr at amino acid 9 is reverted back to wild type by a PCR reaction. The second expression vector pDSY2 is identical to pDSY1 except for this single base change. Three  
15 different *A. oryzae* strains and several *A. niger* strains are cotransformed with pDSY2 and either pTOC90 (WO 91/17243) which carries the *A. nidulans amdS* gene or pSO2 which carries the *A. oryzae pyrG* gene.

- Expression of laccase is observed in all hosts tested,  
20 with both DSY1 and DSY2. Yields range from 0.1-12.0  $\Delta$ abs/min/ml, with highest yields being observed with *A. niger* strains.

- A construct pDSY10 is made which contains the TAKA  
25 promoter, laccase full-length cDNA including its own signal sequence and the AMG terminator. A 200 bp BamHI/AgeI fragment which has a BamHI site immediately 5' to the ATG of the initiation codon and an AgeI site at the same position as in pDSY1 is PCR amplified using *lacI* as template. A  
30 MluI/HindIII fragment is PCR amplified using pDSY2 as template and begins with the MluI site present in the cDNA and ends with a HindII site directly 3' to the stop codon of laccase. The above two fragments and the AgeI/MluI fragment



from pDSY2 are ligated into pHD414 to yield pDSY10 (Figure 8).

The vector pHD414 used in expression of laccase is a derivative of the plasmid p775 (EP 238 023). In contrast to this plasmid, pHD414 has a string of unique restriction sites between the TAKA promoter and the AMG terminator. The plasmid is constructed by removal of an approximately 200 bp long fragment (containing undesirable RE sites) at the 3' end of the terminator, and subsequent removal of an approximately 250 bp long fragment at the 5' end of the promoter, also containing undesirable sites. The 200 bp region is removed by cleavage with NarI (positioned in the pUC vector) and XbaI (just 3' to the terminator), subsequent filling in the generated ends with Klenow DNA polymerase + dNTP, purification of the vector fragment on a gel and religation of the vector fragment. This plasmid is called pHD413. pHD413 is cut with StuI (positioned in the 5' end of the promoter) and PvuII (in the pUC vector), fractionated on gel and religated, resulting in pHD414. Cotransformation of *A. oryzae* HowB104 and *A. niger* Bo-1 are done using pToC90 for selection. Yields in shake flask are comparable to those seen with pDSY2.

## 2. Expression in fermentors

A 1 ml aliquot of a spore suspension of *Aspergillus niger* transformant Bo-1-pDSY10-4 (approximately  $10^9$  spores/ml) is added aseptically to a 500 ml shake flask containing 100 ml of sterile shake flask medium (glucose, 75g/l; soya meal, 20 g/l;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2g/l;  $\text{KH}_2\text{PO}_4$ , 10g/l;  $\text{K}_2\text{SO}_4$ , 2g/l;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.5 g/l; Citric acid, 2g/l; yeast extract, 10g/l; trace metals [ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 14.3 g/l;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 2.5 g/l;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.5 g/l;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 13.8 g/l,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 8.5 g/l; citric acid, 3.0 g/l], 0.5 ml/l; urea, 2g/l, made with tap water and adjusted to pH 6.0 before autoclaving), and incubated at 37°C on a rotary shaker at 200 rpm for 18

hours. 50 ml of this culture is aseptically transferred to a 3 liter fermentor containing 1.8 liters of the fermentor media (maltodextrin MD01 300 g/l;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2g/l;  $\text{KH}_2\text{PO}_4$ , 2g/l; citric acid 2g/l;  $\text{K}_2\text{SO}_4$ , 2.7 g/l;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2g/l; trace metals, 0.5 ml/l; pluronic antifoam, 1ml/l; made with tap water and pH adjusted to 6.0 before autoclaving). The fermentor temperature is maintained at 34°C by the circulation of cooling water through the fermentor jacket. Sterile air is sparged through the fermentor at a rate of 1.8 liter/min (1v/v/m). The agitation rate is maintained at 800 rpm for the first 24 hours after inoculation and at 1300 rpm for the remainder of the fermentation. The pH of the fermentation is kept at 4.0 by the automatic addition of 5N NaOH or  $\text{H}_3\text{PO}_4$ . Sterile feed (urea, 50 g/l; pluronic antifoam, 1.5 ml/l, made up with distilled water and autoclaved) is added to the fermentor by use of a peristaltic pump. The feed rate profile during the fermentation is as follows: 40 g of feed is added initially before inoculation; after inoculation, feed is at a constant rate of 2.5 g/l h.

Copper is made as a 400X stock in water or a suitable buffer, filter sterilized and added aseptically to the tank to a final level of 0.5 mM. Samples for enzyme activity determination are withdrawn and filtered through Miracloth to remove mycelia. These samples are assayed for laccase activity by a LACU assay. Laccase activity is found to increase continuously during the course of the fermentation, with a value of approximately 55 LACU/ml is achieved after 190 hours. This corresponds to approximately 350mg/l of recombinant laccase expressed.

#### IV. PURIFICATION OF RECOMBINANT LACCASE

##### MATERIALS AND METHODS

##### 1. Materials.

Chemicals used as buffers and substrates are commercial products of at least reagent grade. Endo/N-glycosidase G is

from Boehringer-Mannheim. Chromatography is performed on either a Pharmacia's FPLC or a conventional open column low pressure system. Spectroscopic assays are conducted on a Shimadzu PC160 spectrophotometer.

5        2. Purification

         (a) DSY2

         2.8 liters cheese-cloth filtered broth(pH 7, 19mS) obtained from an A. oryzae pDSY2 transformant as described above is filtered on 0.45  $\mu$  Corning filter and concentrated  
10 on Spiral Concentrator(Amicon) with S1Y30 membrane to 200ml. The concentrate pH is adjusted to 7.5, diluted with 4.8 l water to achieve 1.2 mS, and concentrated on S1Y30 to 200ml. 50ml of this broth solution is applied onto a Q-Sepharose column(XK16, 34ml gel), pre-equilibrated with 10mM Tris, pH  
15 7.5, 0.7 mS(Buffer A). The blue laccase band that migrates slowly during loading is eluted by a linear gradient of Buffer B(Buffer A plus 0.5 M NaCl). 24 ml of pooled laccase fractions are concentrated on Centricon-100(Amicon) to 4.5 ml and applied onto a Superdex 200 column(HiLoad 16/60, 120  
20 ml gel). During the development with Buffer C(Buffer A plus 0.15 M NaCl, 14.4 mS), the blue laccase fractions elute followed by brownish contaminant fractions. Only the first half of the elution band(detected by Abs<sub>600</sub>) show a high laccase to contaminant ratio and are pooled. The pooled  
25 fractions are dialyzed in 10mM Bis-Tris, pH 6.8, 0.6mS(Buffer D), applied onto a Mono-Q column(Mono-Q 5/5, 1ml) equilibrated with Buffer D, and eluted with Buffer E(Bufer D plus 0.5 M NaCl) using a linear gradient. The laccase fractions, which ome out round 27% Buffer E, are  
30 pure as judged by SDS-PAGE. At each step, the laccase fractions are routinely checked by ABTS oxidation, SDS-PAGE, and Western Blot.

         (b) DSY10

2.8 liters cheese-cloth filtered broth(pH 7.3, 24mS) obtained from HowB104-pDSY10 is filtered on Whatman #2 paper and concentrated on Spiral Concentrator(Amicon) with S1Y100 membrane to 210ml. The concentrate pH is diluted with  
5 water to achieve 1.2 mS, and concentrated on S1Y100 to 328 ml. This broth solution is applied onto a Q-Sepharose column(XK26, 120 ml gel), pre-equilibrated with 10mM Tris, pH 7.5, 0.7 mS(Buffer A). The blue laccase band that migrates slowly during loading is eluted by a linear  
10 gradient of Buffer B(Buffer A plus 2 M NaCl). 120 ml of pooled laccase fractions are diluted with water to achieve 1.1mS and then concentrated on SIY100 to 294 ml and applied onto a Mono-Q column(HiLoad 16/10, 40 ml gel) pre-equilibrated with Buffer A. The laccase slowly passes  
15 through the column during loading and washing with Buffer A. The pooled fractions which have a pH reading of 5.6, are loaded on a Mono-Q column(HiLoad 16/10, 40 ml gel), pre-equilibrated with Buffer C(10mM MES, pH 5.5, 0.1 mS). The laccase fractions elute by a very shallow gradient of Buffer  
20 D(Buffer C + 1M NaCl). Enzymatic assays are conducted as described above.

### 3. Protein analysis

Total amino acid analysis, N-terminal sequencing, deglycosylation, SDS-PAGE, IEF, and Western blots are  
25 performed as decribed above.

## **B. RESULTS AND DISCUSSION**

### 1. Purification and Characterization

Overall a 256-fold purification and a yield of 37% are achieved for DSY10, and a 246-fold purification and a yield  
30 of 14% are achieved for DSY2. In terms of electrophoretic pattern, spectral properties and activity, purified DSY2 and DSY10 are indistinguishable. Purified recombinant laccases behave as a dimer on gel filtration, and exhibit subunit molecular weight which is somewhat larger than that of the

wild type laccase, indicating a post-translational processing in *A. oryzae* that results in the extra glycosylation on the recombinants. Deglycosylation has confirmed the difference in mass arising from extra  
5 sugars (Table 3).

Table 3. Molecular and spectral properties of recombinant and wild-type laccase

5	MW, kDa		Carbohydrate	pI	$\lambda_{\max}$ , nm ( $\epsilon$ , l/g*cm)
	Native	subunit	w/w%		
WT	~130	~63	~7	3.5	275(1.8) 615(0.12)
Rec.	~130	~67	~13	3.5	275(1.7) 615(0.11)

10

The spectra of the purified laccases have maxima of 615 nm and 275, with the ratio of absorbance at 275 nm to that at 615 nm being 16, indicating one Type I Cu per subunit. The ratio of absorbance at 330nm to that at 615nm is 1.0, close to the 0.75 value of *Rhus vernicefera* laccase, suggesting the presence of one Type II and two Type III copper ions per subunit. The extinction coefficient determined by amino acid analysis is 1.71/(g\*cm),

### 3. Activity

20 The laccase activity is measured by syringaldazine and ABTS oxidations. Expressed per  $A_{275}$ , the laccase has a value of 83 for LACU. Expressed per mg, it has a LACU of 141. The pH profile of the laccase is provided in Figure 9.

## 25 V. USE OF POLYPORUS LACCASE TO DYE HAIR

The dyeing effect of *Polyporus pinsitus* laccase is tested and compared to the dyeing effect of 3%  $H_2O_2$  on various dye precursors (listed below) and further on 0.1% p-phenylenediamine compared with a number of modifiers.

30

### Materials:

#### Dye precursors:

0.1 % p-phenylene-diamine in 0.1 M K-phosphate buffer, pH 7.0. (pPD)

0.1 % p-toluylene-diamine in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % chloro-p-phenylenediamine in 0.1 M K-phosphate buffer, pH 7.0.

5 0.1 % p-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % o-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % 3,4-diaminotoluene in 0.1 M K-phosphate, buffer pH 7.0.

10 Modifiers:

0.1 % m-phenylene-diamine in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % 2,4-diaminoanisole in 0.1 M K-phosphate buffer, pH 7.0.

15 0.1 %  $\alpha$ -naphthol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % hydroquinone in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % pyrocatechol in 0.1 M K-phosphate buffer, pH 7.0.

0.1% resorcinol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % 4-chlororesorcinol in 0.1 M K-phosphate buffer, pH  
20 7.0.

When a modifier is used, the dye precursor p-phenylene-diamine is combined with one of the above indicated modifiers so that the final concentration in the dyeing

25 solution is 0.1 % with respect to precursor and 0.1 % with respect to modifier. The enzyme used is a recombinant laccase from *Polyporus pinisitus*, at a concentration of 10 LACU/ml.

30 Other solutions used in the process are 3% H<sub>2</sub>O<sub>2</sub> (in the final dye solution), and a commercial shampoo.

The quantitative color of the hair tresses is determined on a Datacolor Textflash 2000 (CIE-Lab) by the use of

CIE-Lab parameters  $L^*$  ("0"=black and "100"=white) combined with  $a^*$  ("-"=green and "+"=red).  $\Delta L^*$  and  $\Delta a^*$  are the delta values of  $L^*$  and  $a^*$ , respectively, of a sample when compared to  $L^*$  and  $a^*$  of untreated hair. The Light fastness is  
5 determined under a day light bulb (D65) at 1000 LUX.

Hair tresses of blond European hair (1 gram) are used.  
4 ml dye precursor solution (including modifier) is mixed with 1 ml laccase or 1 ml  $H_2O_2$  on a Whirley mixer, applied to  
10 the hair tresses and kept at 30°C for 60 minutes. The hair tresses are then rinsed with running water, combed, and air dried.

The results of the dyeing effect test are displayed below in  
15 Table 4-6 and further in the graphs in Figures 10 to 12.



Table 4

Sample no.	Sample ID	L*	a*	DL*	Da*
	Untreated blond hair	72.25	2.42		
1	p-phenylenediamine (Reference)	62.85	4.03	-9.41	1,61
2	p-phenylenediamine + Laccase	28.70	0.33	-43.56	-2,10
3	p-phenylenediamine + 3% H <sub>2</sub> O <sub>2</sub>	21.88	2.04	-50.37	-0,39
4	p-Toluylenediamine (Reference)	58.14	4.34	-14.11	1.92
5	p-Toluylenediamine + Laccase	36.70	8.09	-35.56	5.67
6	p-Toluylenediamine + 3% H <sub>2</sub> O <sub>2</sub>	42.30	6.24	-29.95	3.81
7	chloro-p-phenylenediamine (Reference)	69.82	3.23	-2.43	0.81
8	chloro-p-phenylenediamine + Laccase	35.58	9.36	-36.68	6.93
9	chloro-p-phenylenediamine + 3% H <sub>2</sub> O <sub>2</sub>	45.42	9.59	-26.84	7.17
10	p-aminophenol (Reference)	66.62	5.03	-5.63	2.61
11	p-aminophenol + Laccase	42,42	7.38	-29,84	4.95
12	p-aminophenol + 3% H <sub>2</sub> O <sub>2</sub>	50.54	9.42	-21.72	7.26
13	o-aminophenol (Reference)	69.39	4.82	-2.89	2.39
14	o-aminophenol + Laccase	60.20	12.92	-12.05	10.50
15	o-aminophenol + 3% H <sub>2</sub> O <sub>2</sub>	63.49	10.38	-8.77	7.96
16	3,4-diaminotoluene (Reference)	69.62	3.57	-2.63	1.15
17	3,4-diaminotoluene + Laccase	39.51	3.15	-32.74	0.73
18	3,4-diaminotoluene + 3% H <sub>2</sub> O <sub>2</sub>	59.32	4.16	-12.94	1.74

L\*: 0=black, 100=white      a\*: -=green, +=red

Table 5

Sample no.	Sample ID	L*	a*	DL*	Da*
	Untreated blond hair	72.25	2.42		
19	p-phenylenediamine+ m-phenylenediamin (Reference)	58.82	0.43	-13,44	-1,99
20	p-phenylenediamine + m-phenylenediamin + Laccase	27.20	0.83	-45,05	-1,59
21	p-phenylenediamine + m-phenylenediamine + 3% H2O2	16.96	0.13	-55,29	-2,59
22	p-phenylenediamine + 2,4 - diaminoanisole (Reference)	35.37	-0.02	-36,89	-2,45
23	p-phenylenediamine + 2,4 - diaminoanisole + Laccase	24.56	2.99	-47,70	0,57
24	p-phenylenediamine + 2,4-diaminoanisole + 3% H2O2	15.06	2.21	-57,20	-0,21
25	p-phenylenediamine + $\alpha$ -naphthol (Reference)	54.33	2.54	-17,93	0,12
26	p-phenylenediamine + $\alpha$ -naphthol + Laccase	29.53	4.03	-42,72	1,60
27	p-phenylenediamine + $\alpha$ -naphthol + 3% H2O2	19.58	3.90	-52,68	1,47
28	p-phenylenediamine + hydroquinone (Reference)	53.25	4.08	-19,01	1,65
29	p-phenylenediamine + hydroquinone + Laccase	40.48	5.00	-31,77	2,58
30	p-phenylenediamine + hydroquinone + 3% H2O2	29.06	4.96	-43,20	2,53

L\*: 0=black, 100=white      a\*: -=green, +=red

Table 6

Sample no.	Sample ID	L*	a*	DL*	Da*
	Untreated blond hair	72.25	2.42		
31	p-phenylenediamine + pyrocatechol (Reference)	53.78	1.68	-18.47	-0.74
32	p-phenylenediamine + pyrocatechol + Laccase	30.77	2.64	-41.49	0.22
33	p-phenylenediamine + pyrocatechol + 3% H <sub>2</sub> O <sub>2</sub>	22.15	3.30	-50.11	0.88
34	p-phenylenediamine + resorcinol (Reference)	62.12	4.23	-10.14	1.81
35	p-phenylenediamine + resorcinol + Laccase	36.14	2.91	-36.11	0.49
36	p-phenylenediamine + resorcinol + 3% H <sub>2</sub> O <sub>2</sub>	23.94	3.16	-48.31	0.74
40	p-phenylenediamine + 4-chlororesorcinol (Reference)	61.18	4.70	-11.07	2.28
41	p-phenylenediamine + 4-chlororesorcinol + Laccase	36.00	2.76	-36.26	0.34
42	p-phenylenediamine + 4-chlororesorcinol + 3% H <sub>2</sub> O <sub>2</sub>	22.63	2.60	-49.63	0.18

L\*: 0=black, 100=white    a\*: -=green, +=red

The oxidative hair dyeing is carried out as described above, except that 50 LACU/ml *Polyporus pinsitus* laccase was used.

To test wash stability, the dyed hair tresses are wetted and washed for 15 seconds with 50 µl of commercial  
5 shampoo, and rinsed with water for 1 minute. The hair tresses are washed up to 20 times.

The results of the hair wash test are displayed in figure 13. It can be seen in figure 13 that the wash stability of hair washed up to 20 times is excellent, when  
10 using *Polyporus pinsitus* laccase for oxidative dyeing.

To test light fastness, tresses of blond european hair are used for testing the light fastness of hair dyed using *Polyporus pinsitus* laccase in comparison to hair dyed using H<sub>2</sub>O<sub>2</sub>. p-phenylene-diamine is the dye precursor. The dyeing of  
15 the hair is carried out as described above. One hair tress is kept dark, while an other is kept at day light (i.e. under a day light bulb (D65)), at approximately 1000 LUX) for up to 275 hours. The CIE-Lab-values are determined immediately after the dyeing of the hair, and further during  
20 exposure to day light.

The results of the test are displayed in figure 14. Figure 14 shows that the hair dyed with p-phenylene-diamine using *Polyporus pinsitus* laccase has the same light fastness as hair dyed using H<sub>2</sub>O<sub>2</sub>.

25

#### Deposit of Biological Materials

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural  
30 Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria,

Illinois, 61604 on May 25, 1994 and given the following accession numbers.

	<u>Deposit</u>	<u>Accession Number</u>
	<i>E. coli</i> DH5 $\alpha$ containing	NRRL B-21263
5	pDSY22(41GEN; an ~3.0 kb EcoRI insert)	
	<i>E. coli</i> DH5 $\alpha$ containing	NRRL B-21268
	pDSY23(41GEN; an ~4.5 kb MluI insert; insert contains a small portion of the EcoRI fragment of pDSY22 and sequences	
10	5' to the EcoRI fragment)	
	<i>E. coli</i> XL-1 Blue containing	NRRL B-21264
	pDSY21(31GEN; an ~7.7 kb EcoRI/BamHI insert)	
	<i>E. coli</i> XL-1 Blue containing	NRRL B-21265
15	pDSY18(21GEN; an ~8.0 kb BamHI insert)	
	<i>E. coli</i> DH5 $\alpha$ containing	NRRL B-21266
	pDSY19(23GEN; an ~4 kb HindIII insert)	
	<i>E. coli</i> DH5 $\alpha$ containing	NRRL B-21267
	pDSY20(24GEN; an ~8.5 kb EcoRI insert)	

20

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Novo Nordisk Biotech, Inc.  
(B) STREET: 1445 Drew Avenue  
(C) CITY: Davis, California  
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(E) POSTAL CODE (ZIP): 95616-4880  
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(G) TELEFAX: +45 4449 3256  
(F) TELEX: 37304

(ii) TITLE OF INVENTION: PURIFIED POLYPORUS LACCASES AND  
NUCLEIC ACIDS ENCODING SAME

(iii) NUMBER OF SEQUENCES: 10

## (iv) CORRESPONDENCE ADDRESS:

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(C) CITY and STATE: New York, New York  
(D) COUNTRY: U.S.A.  
(E) ZIP: 10174-6401

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: to be assigned  
(B) FILING DATE: 15-June-1995

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/265,534  
(B) FILING DATE: 24-June-1994

## (viii) ATTORNEY/AGENT INFORMATION:

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## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 212 867 0123  
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## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2418 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vi) ORIGINAL SOURCE:  
(A) ORGANISM: Polyporus pinsitus
- (ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 414..464
- (ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 534..589
- (ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 710..764
- (ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 879..934
- (ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 1001..1050
- (ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 1147..1197
- (ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 1354..1410
- (ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 1609..1662
- (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: join (413..465, 533..590, 709..765, 878..935, 1000..1051, 1146..1198, 1353..1411, 1608..1663)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGATTCTGA CACCGGTGCA ATCTTGACAC TGTACCAACC GGGCAAGTCT CGTCCTTGGT	60
TCTCGGGGACT GGCGCCGGT CGCTACCCCT TGGTCATTCA CTCTACCAGA GCGCTGGCTT	120
CGCCGAGGTA TAAAGGATGT TGCGCGACAC CCTCAACACC CCAACTCAAG CCCCACTTGA	180
GCTTTTCCGA GATCCTCCAC ATACCACTCA CTACTTTCAA GTTCTTCAAC ATG TCG AGG	239
Met Ser Arg	
1	
TTT CAC TCT CTT CTC GCT TTC GTC GTT GCT TCC CTT ACG GCT GTG GCC	287
Phe His Ser Leu Leu Ala Phe Val Val Ala Ser Leu Thr Ala Val Ala	
5 10 15	
CAC GCT GGT ATC GGT CCC GTC GCC GAC CTA ACC ATC ACC AAC GCA GCG	335
His Ala Gly Ile Gly Pro Val Ala Asp Leu Thr Ile Thr Asn Ala Ala	
20 25 30 35	
GTC AGC CCC GAC GGG TTT TCT CGC CAG GCC GTC GTC GTG AAC GGC GGC	383
Val Ser Pro Asp Gly Phe Ser Arg Gln Ala Val Val Val Asn Gly Gly	
35 40 45	
ACC CCT GGC CCT CTC ATC ACG GGT AAC ATG GTTCGTCTCG GCTCGCACTA	433
Thr Pro Gly Pro Leu Ile Thr Gly Asn Met	
50 55	
GGGGGTTCTA TCGTTCCTGA CGTTGTTGGA G GGG GAT CGC TTC CAG CTC AAT GTC ATC	491

	Gly Asp Arg Phe Gln Leu Asn Val Ile	
	60 65	
GAC AAC CTT ACC AAC CAC ACG ATG GTG AAG AGC ACG AGT ATT GTGAGCTGCT	543	
Asp Asn Leu Thr Asn His Thr Met Val Lys Ser Thr Ser Ile		
70 75		
ATTTCTCCGG ACGGGGCTTC ATTGTGCTAA TAATCGTCGT GTGCAG CAC TGG CAC GGT	601	
His Trp His Gly		
80		
TTC TTC CAG AAG GGT ACC AAC TGG GCC GAC GGT CCC GCC TTC ATC AAC	649	
Phe Phe Gln Lys Gly Thr Asn Trp Ala Asp Gly Pro Ala Phe Ile Asn		
85 90 95		
CAG TGC CCG ATC TCA TCT GGT CAC TCG TTC CTG TAC GAC TTC CAG GTT	697	
Gln Cys Pro Ile Ser Ser Gly His Ser Phe Leu Tyr Asp Phe Gln Val		
100 105 110 115		
CCT GAC CAG GCT GTAAGTACGG TCGTTATGGA GTATACTGCG CATTGCTAAA	749	
Pro Asp Gln Ala		
CCACATGGTG AACAG GGT ACC TTC TGG TAT CAC AGT CAC TTG TCT ACG CAG	800	
Gly Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln		
120 125 130		
TAC TGT GAT GGT TTG AGG GGT CCG TTC GTT GTT TAC GAC CCG AAT GAC	848	
Tyr Cys Asp Gly Leu Arg Gly Pro Phe Val Val Tyr Asp Pro Asn Asp		
135 140 145		
CCG GCC GCC GAC CTG TAC GAC GTC GAC AAC GTAAGGACGA ATTCGAACCG	898	
Pro Ala Ala Asp Leu Tyr Asp Val Asp Asn		
150 155		
TAAATACTTG CTTACTGATA CTTCTCGATG AATTAG GAC GAC ACT GTC ATT	949	
Asp Asp Thr Val Ile		
160		
ACC CTT GTG GAT TGG TAC CAC GTC GCC GCG AAG CTG GGC CCC GCA TTC	997	
Thr Leu Val Asp Trp Tyr His Val Ala Ala Lys Leu Gly Pro Ala Phe		
165 170 175		
CCT GTAAGTCCAT GAGTATTCTG CTGTTGAATC TGTCTTAACT GTGCATATCA CTC	1053	
Pro Leu		
180		
GGC GCC GAC GCC ACC CTC ATC AAC GGT AAG GGA CGC TCC CCC AGC ACG	1101	
Gly Ala Asp Ala Thr Leu Ile Asn Gly Lys Gly Arg Ser Pro Ser Thr		
185 190 195		
ACC ACC GCG GAC CTC TCA GTT ATC AGC GTC ACC CCG GGT AAA CGC	1146	
Thr Thr Ala Asp Leu Ser Val Ile Ser Val Thr Pro Gly Lys Arg		
200 205 210		
GTATGCTATA TCTTATCTTA TCTGATGGCA TTTCTCTGAG ACATTCTCCA G	1197	
TAC CGT TTC CGC CTG GTG TCC CTG TCG TGC GAC CCC AAC TAC ACG TTC	1245	
Tyr Arg Phe Arg Leu Val Ser Leu Ser Cys Asp Pro Asn Tyr Thr Phe		
215 220 225		
AGC ATC GAT GGT CAC AAC ATG ACG ATC ATC GAG ACC GAC TCA ATC AAC	1293	
Ser Ile Asp Gly His Asn Met Thr Ile Ile Glu Thr Asp Ser Ile Asn		
230 235 240		
ACG GCG CCC CTC GTC GTC GAC TCC ATT CAG ATC TTC GCC GCC CAG CGT	1341	
Thr Ala Pro Leu Val Val Ser Ile Gln Ile Phe Ala Ala Gln Arg		
245 250 255		
TAC TCC TTC GTG GTAAGTTCGA TTCATCCTCT AACGTTGGTC GCTGTTAGTG	1393	



Tyr Ser Phe Val  
260

ATCGTATGGT CATGTAG CTC GAG GCC AAC CAG GCC GTC GAC AAC TAC TGG 1443  
Leu Glu Ala Asn Gln Ala Val Asp Asn Tyr Trp  
265 270

ATT CGC GCC AAC CCG AAC TTC GGT AAC GTC GGG TTC ACC GGC GGC ATT 1491  
Ile Arg Ala Asn Pro Asn Phe Gly Asn Val Gly Phe Thr Gly Gly Ile  
275 280 285 290

AAC TCG GCT ATC CTC CGC TAC GAT GGT GCC GCT GCC GTG GAG CCC ACC 1539  
Asn Ser Ala Ile Leu Arg Tyr Asp Gly Ala Ala Val Glu Pro Thr  
295 300 305

ACA ACG CAA ACC ACG TCG ACT GCG CCG CTC AAC GAG GTC AAC CTG CAC 1587  
Thr Thr Gln Thr Ser Thr Ala Pro Leu Asn Glu Val Asn Leu His  
310 315 320

CCG CTG GTT ACC ACC GCT GTG GTATGTAATA TTGTCGGTAA TGTAAATACAT 1638  
Pro Leu Val Thr Thr Ala Val  
325

TGTTGCTGAC CTCGACCCCC ACAG CCT GGC TCG CCC GTC GCT GGT GGT GTC 1689  
Pro Gly Ser Pro Val Ala Gly Gly Val  
330 335

GAC CTG GCC ATC AAC ATG GCG TTC AAC TTC AAC GGC ACC AAC TTC TTC 1737  
Asp Leu Ala Ile Asn Met Ala Phe Asn Phe Asn Gly Thr Asn Phe Phe  
340 345 350

ATC AAC GGC ACG TCT TTC ACG CCC CCG ACC GTG CCT GTC CTG CTC CAG 1785  
Ile Asn Gly Thr Ser Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln  
355 360 365 370

ATC ATC AGC GGC GCG CAG AAC GCG CAG GAC CTC CTG CCC TCC GGT AGC 1833  
Ile Ile Ser Gly Ala Gln Asn Ala Gln Asp Leu Leu Pro Ser Gly Ser  
375 380 385

GTC TAC TCG CTT CCC TCG AAC GCC GAC ATC GAG ATC TCC TTC CCC GCC 1881  
Val Tyr Ser Leu Pro Ser Asn Ala Asp Ile Glu Ile Ser Phe Pro Ala  
390 395 400

ACC GCC GCC GCC CCC GGT GCG CCC CAC CCC TTC CAC TTG CAC GGG CAC 1929  
Thr Ala Ala Ala Pro Gly Ala Pro His Pro Phe His Leu His Gly His  
405 410 415

GCG TTC GCG GTC GTC CCG AGC GCC GGC AGC ACG GTT TAC AAC TAC GAC 1977  
Ala Phe Ala Val Val Arg Ser Ala Gly Ser Thr Val Tyr Asn Tyr Asp  
420 425 430

AAC CCC ATC TTC CCG GAC GTC GTC AGC ACG GGG ACG CCT GCG GCC GGT 2025  
Asn Pro Ile Phe Arg Asp Val Val Ser Thr Gly Thr Pro Ala Ala Gly  
435 440 445 450

GAC AAC GTC ACC ATC CCG TTC CCG ACC GAC AAC CCC GGC CCG TGG TTC 2073  
Asp Asn Val Thr Ile Arg Phe Arg Thr Asp Asn Pro Gly Pro Trp Phe  
455 460 465

CTC CAC TGC CAC ATC GAC TTC CAC CTC GAG GCC GGC TTC GCC GTC GTG 2121  
Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Val Val  
470 475 480

TTC GCG GAG GAC ATC CCC GAC GTC GCG TCG GCG AAC CCC GTC CCC CAG 2169  
Phe Ala Glu Asp Ile Pro Asp Val Ala Ser Ala Asn Pro Val Pro Gln  
485 490 495

GCG TGG TCC GAC CTC TGT CCG ACC TAC GAC GCG CTC GAC CCG AGC GAC 2217

Ala Trp Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Pro Ser Asp  
500 505 510

CAG TAAATGGCTT GCGCCGGTCG ATGATAGGAT ATGGACGGTG AGTTCGCACT 2270  
Gln  
515

TGCAATACGG ACTCTCGCCT CATTATGGTT ACACACTCGC TCTGGATCTC TCGCCTGTCG 2330

ACAGAACAAA CTTGTATAAT TCGCTTAATG GTTGAAACAA ATGGAATATT GGGGTACTAT 2390

GCACGCATCT CGCTGGGTGA GCTTTCGT 2418

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 520 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Polyporus pinsitus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ser Arg Phe His Ser Leu Leu Ala Phe Val Val Ala Ser Leu Thr  
1 5 10 15  
Ala Val Ala His Ala Gly Ile Gly Pro Val Ala Asp Leu Thr Ile Thr  
20 25 30  
Asn Ala Ala Val Ser Pro Asp Gly Phe Ser Arg Gln Ala Val Val Val  
35 40 45  
Asn Gly Gly Thr Pro Gly Pro Leu Ile Thr Gly Asn Met Gly Asp Arg  
50 55 60  
Phe Gln Leu Asn Val Ile Asp Asn Leu Thr Asn His Thr Met Val Lys  
65 70 75 80  
Ser Thr Ser Ile His Trp His Gly Phe Phe Gln Lys Gly Thr Asn Trp  
85 90 95  
Ala Asp Gly Pro Ala Phe Ile Asn Gln Cys Pro Ile Ser Ser Gly His  
100 105 110  
Ser Phe Leu Tyr Asp Phe Gln Val Pro Asp Gln Ala Gly Thr Phe Trp  
115 120 125  
Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro  
130 135 140  
Phe Val Val Tyr Asp Pro Asn Asp Pro Ala Ala Asp Leu Tyr Asp Val  
145 150 155 160  
Asp Asn Asp Asp Thr Val Ile Thr Leu Val Asp Trp Tyr His Val Ala  
165 170 175  
Ala Lys Leu Gly Pro Ala Phe Pro Leu Gly Ala Asp Ala Thr Leu Ile  
180 185 190  
Asn Gly Lys Gly Arg Ser Pro Ser Thr Thr Thr Ala Asp Leu Ser Val  
195 200 205  
Ile Ser Val Thr Pro Gly Lys Arg Tyr Arg Phe Arg Leu Val Ser Leu

210	215	220
Ser Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Met Thr		
225	230	235 240
Ile Ile Glu Thr Asp Ser Ile Asn Thr Ala Pro Leu Val Val Asp Ser		
	245	250 255
Ile Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val Leu Glu Ala Asn		
	260	265 270
Gln Ala Val Asp Asn Tyr Trp Ile Arg Ala Asn Pro Asn Phe Gly Asn		
	275	280 285
Val Gly Phe Thr Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr Asp Gly		
	290	295 300
Ala Ala Ala Val Glu Pro Thr Thr Thr Gln Thr Thr Ser Thr Ala Pro		
	305	310 315 320
Leu Asn Glu Val Asn Leu His Pro Leu Val Thr Thr Ala Val Pro Gly		
	325	330 335
Ser Pro Val Ala Gly Gly Val Asp Leu Ala Ile Asn Met Ala Phe Asn		
	340	345 350
Phe Asn Gly Thr Asn Phe Phe Ile Asn Gly Thr Ser Phe Thr Pro Pro		
	355	360 365
Thr Val Pro Val Leu Leu Gln Ile Ile Ser Gly Ala Gln Asn Ala Gln		
	370	375 380
Asp Leu Leu Pro Ser Gly Ser Val Tyr Ser Leu Pro Ser Asn Ala Asp		
	385	390 395 400
Ile Glu Ile Ser Phe Pro Ala Thr Ala Ala Ala Pro Gly Ala Pro His		
	405	410 415
Pro Phe His Leu His Gly His Ala Phe Ala Val Val Arg Ser Ala Gly		
	420	425 430
Ser Thr Val Tyr Asn Tyr Asp Asn Pro Ile Phe Arg Asp Val Val Ser		
	435	440 445
Thr Gly Thr Pro Ala Ala Gly Asp Asn Val Thr Ile Arg Phe Arg Thr		
	450	455 460
Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu		
	465	470 475 480
Glu Ala Gly Phe Ala Val Val Phe Ala Glu Asp Ile Pro Asp Val Ala		
	485	490 495
Ser Ala Asn Pro Val Pro Gln Ala Trp Ser Asp Leu Cys Pro Thr Tyr		
	500	505 510
Asp Ala Leu Asp Pro Ser Asp Gln		
	515	520

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2880 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ix) FEATURE:
- (A) NAME/KEY: intron

(B) LOCATION: 544..592

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 837..899

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1014..1066

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1133..1187

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1284..1342

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1752..1815

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1873..1928

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 2136..2195

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: join(364..543, 593..661, 716..835, 900..1013,  
1067..1132, 1188..1283, 1343..1498, 1554..1751,  
1816..1872, 1929..2135, 2196..2489)

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 662..715

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1499..1553

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCGGCGCACA AACCGTGGGA GCCAACACAC TCCCGTCCAC TCTCACA CTG GCCAGATTCG	60
CGCGACCGCC GCCTTTCAGG CCCAAACAGA TCTGGCAGGT TTCGATGGCG CACGCCGCCG	120
TGCCTGCCGG ATTCAATTGT GCGCCAGTCG GGCATCCGGA TGGCTCTACC AGCGCGGTTC	180
ACTGGAAGAG AACACCGAGG TCATGCATTC TGGCCAAGTG CGGCCAAAGG ACCGCTCGCT	240
GGTGCGGATA CTAAAGGGC GCGCGGGGGA GGCCTGTCTA CCAAGCTCAA GCTCGCCTTG	300
GGTTCCAGT CTCCGCCACC CTCCTCTTCC CCCACACAGT CGCTCCATAG CACCGTCGGC	360
GCC ATG GGT CTG CAG CGA TTC AGC TTC TTC GTC ACC CTC GCG CTC GTC	408
Met Gly Leu Gln Arg Phe Ser Phe Phe Val Thr Leu Ala Leu Val	
1 5 10 15	
GCT CGC TCT CTT GCA GCC ATC GGG CCG GTG GCG AGC CTC GTC GTC GCG	456
Ala Arg Ser Leu Ala Ala Ile Gly Pro Val Ala Ser Leu Val Val Ala	
20 25 30	
AAC GCC CCC GTC TCG CCC GAC GGC TTC CTT CGG GAT GCC ATC GTG GTC	504
Asn Ala Pro Val Ser Pro Asp Gly Phe Leu Arg Asp Ala Ile Val Val	
35 40 45	

AAC GGC GTG GTC CCT TCC CCG CTC ATC ACC GGG AAG AAG GTCGGCGTGT Asn Gly Val Val Pro Ser Pro Leu Ile Thr Gly Lys Lys 50 55 60	553
TCGTCGTCGT CCTACTCCTT TGCTGACAGC GATCTACAG GGA GAC CGC TTC CAG Gly Asp Arg Phe Gln 65	607
CTC AAC GTC GTC GAC ACC TTG ACC AAC CAC AGC ATG CTC AAG TCC ACT Leu Asn Val Val Asp Thr Leu Thr Asn His Ser Met Leu Lys Ser Thr 70 75 80	655
AGT ATC GTAAGTGTA CGATCCGAAT GTGACATCAA TCGGGGCTAA TTAACCGCGC Ser Ile	711
ACAG CAC TGG CAC GGC TTC TTC CAG GCA GGC ACC AAC TGG GCA GAA GGA His Trp His Gly Phe Phe Gln Ala Gly Thr Asn Trp Ala Glu Gly 85 90 95	760
CCC GCG TTC GTC AAC CAG TGC CCT ATT GCT TCC GGG CAT TCA TTC CTG Pro Ala Phe Val Asn Gln Cys Pro Ile Ala Ser Gly His Ser Phe Leu 100 105 110	808
TAC GAC TTC CAT GTG CCC GAC CAG GCA GTAAGCAGGA TTTTCTGGGG Tyr Asp Phe His Val Pro Asp Gln Ala 115 120	855
TCCCCGTGTG ATGCAATGTT CTCATGCTCC GACGTGATCG ACAG GGG ACG TTC TGG Gly Thr Phe Trp 125	911
TAC CAC AGT CAT CTG TCT ACG CAG TAC TGT GAC GGG CTG CGG GGG CCG Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro 130 135 140	959
TTC GTC GTG TAC GAC CCC AAG GAC CCG CAC GCC AGC CGT TAC GAT GTT Phe Val Val Tyr Asp Pro Lys Asp Pro His Ala Ser Arg Tyr Asp Val 145 150 155	1007
GAC AAT GTACGTGCGC CACGGAGTAT ATCACACAGC ATGCGTTGAC GTCGGGCCAA Asp Asn 160	1063
CAG GAG AGC ACG GTC ATC ACG TTG ACC GAC TGG TAC CAC ACC GCT GCC Glu Ser Thr Val Ile Thr Leu Thr Asp Trp Tyr His Thr Ala Ala 165 170 175	1111
CGG CTC GGT CCC AAG TTC CCA GTAAGCTCGC AATGGCTTAG TGTTTCACAGG Arg Leu Gly Pro Lys Phe Pro 180	1162
TTCTTTGCTT ATGTTGCTTC GATAG CTC GGC GCG GAC GCC ACG CTC ATC AAC Leu Gly Ala Asp Ala Thr Leu Ile Asn 185 190	1214
GGT CTG GGG CGG TCG GCC TCG ACT CCC ACC GCT GCG CTT GCC GTG ATC Gly Leu Gly Arg Ser Ala Ser Thr Pro Thr Ala Ala Leu Ala Val Ile 195 200 205	1262
AAC GTC CAG CAC GGA AAG CGC GTGAGCATTC TCTTGTATGC CATTTC AATG Asn Val Gln His Gly Lys Arg 210 215	1313
CTTTGTGCTG ACCTATCGGA ACCGCGCAG TAC CGC TTC CGT CTC GTT TCG ATC Tyr Arg Phe Arg Leu Val Ser Ile 220	1366

TCG TGT GAC CCG AAC TAC ACG TTC AGC ATC GAC GGG CAC AAC CTG ACC Ser Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Leu Thr 225 230 235	1414
GTC ATC GAG GTC GAC GGC ATC AAT AGC CAG CCT CTC CTT GTC GAC TCT Val Ile Glu Val Asp Gly Ile Asn Ser Gln Pro Leu Leu Val Asp Ser 240 245 250 255	1462
ATC CAG ATC TTC GCC GCA CAG CGC TAC TCC TTC GTG GTAAGTCCTG Ile Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val 260 265	1508
GCTTGTCGAT GCTCCAAAGT GGCCTCACTC ATATACTTTC GTTAG TTG AAT GCG Leu Asn Ala 270	1562
AAT CAA ACG GTG GGC AAC TAC TGG GTT CGT GCG AAC CCG AAC TTC GGA Asn Gln Thr Val Gly Asn Tyr Trp Val Arg Ala Asn Pro Asn Phe Gly 275 280 285	1610
ACG GTT GGG TTC GCC GGG GGG ATC AAC TCC GCC ATC TTG CGC TAC CAG Thr Val Gly Phe Ala Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr Gln 290 295 300	1658
GGC GCA CCG GTC GCC GAG CCT ACC ACG ACC CAG ACG CCG TCG GTG ATC Gly Ala Pro Val Ala Glu Pro Thr Thr Thr Gln Thr Pro Ser Val Ile 305 310 315	1706
CCG CTC ATC GAG ACG AAC TTG CAC CCG CTC GCG CGC ATG CCA GTG Pro Leu Ile Glu Thr Asn Leu His Pro Leu Ala Arg Met Pro Val 320 325 330	1751
GTATGTCTCT TTTTCTGATC ATCTGAGTTG CCCGTTGTTG ACCGCATTAT GTGTTACTAT	1811
CTAG CCT GGC AGC CCG ACA CCC GGG GGC GTC GAC AAG GCG CTC AAC CTC Pro Gly Ser Pro Thr Pro Gly Gly Val Asp Lys Ala Leu Asn Leu 335 340 345	1860
GCG TTT AAC TTC GTAAGTATCT CTACTACTTA GGCTGGAGGC TCGTCGCTGA Ala Phe Asn Phe 350	1912
TCATACGGTG CTTCAG AAC GGC ACC AAC TTC TTC ATC AAC AAC GCG ACT Asn Gly Thr Asn Phe Phe Ile Asn Asn Ala Thr 355 360	1961
TTC ACG CCG CCG ACC GTC CCG GTA CTC CTC CAG ATT CTG AGC GGT GCG Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala 365 370 375	2009
CAG ACC GCA CAA GAC CTG CTC CCC GCA GGC TCT GTC TAC CCG CTC CCG Gln Thr Ala Gln Asp Leu Leu Pro Ala Gly Ser Val Tyr Pro Leu Pro 380 385 390 395	2057
GCC CAC TCC ACC ATC GAG ATC ACG CTG CCC GCG ACC GCC TTG GCC CCG Ala His Ser Thr Ile Glu Ile Thr Leu Pro Ala Thr Ala Leu Ala Pro 400 405 410	2105
GGT GCA CCG CAC CCC TTC CAC CTG CAC GGT GTATGTTCCC CTGCCTTCCC Gly Ala Pro His Pro Phe His Leu His Gly 415 420	2155
TTCTTATCCC CGAACCAGTG CTCACGTCCG TCCCATCTAG CAC GCC TTC GCG GTC His Ala Phe Ala Val 425	2210
GTT CGC AGC GCG GGG AGC ACC ACG TAT AAC TAC AAC GAC CCG ATC TTC Val Arg Ser Ala Gly Ser Thr Thr Tyr Asn Tyr Asn Asp Pro Ile Phe 430 435 440	2258

CGC GAC GTC GTG AGC ACG GGC ACG CCC GCC GCG GGC GAC AAC GTC ACG 2306  
 Arg Asp Val Val Ser Thr Gly Thr Pro Ala Ala Gly Asp Asn Val Thr  
 445 450 455

ATC CGC TTC CAG ACG GAC AAC CCC GGG CCG TGG TTC CTC CAC TGC CAC 2354  
 Ile Arg Phe Gln Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His  
 460 465 470

ATC GAC TTC CAC CTC GAC GCA GGC TTC GCG ATC GTG TTC GCA GAG GAC 2402  
 Ile Asp Phe His Leu Asp Ala Gly Phe Ala Ile Val Phe Ala Glu Asp  
 475 480 485 490

GTT GCG GAC GTG AAG GCG GCG AAC CCG GTT CCG AAG GCG TGG TCG GAC 2450  
 Val Ala Asp Val Lys Ala Ala Asn Pro Val Pro Lys Ala Trp Ser Asp  
 495 500 505

CTG TGC CCG ATC TAC GAC GGG CTG AGC GAG GCT AAC CAG TGAGCGGAGG 2499  
 Leu Cys Pro Ile Tyr Asp Gly Leu Ser Glu Ala Asn Gln  
 510 515

GCGTGTTGTT GAGCGTAAAG CTCGGGCGTC GACCTGGGGG GTTGAAGGTG TTCTGATTGA 2559

AATGGTCTTT GGGTTTATTT GTTGTTATTC TAACTCGGTT CTCTACGCAA GGACCGAGGA 2619

TTGTATAGGA TGAAGTAACT TCCCTAATGT ATTATGATAT CAATTGACGG AGGCATGGAC 2679

TGCGAAGTGT GTACAATGTG GTAGTGGTCT AGGCCTTGGA GACAAGCTGT GGATTTTCT 2739

TGGGGGATGA AGAGCGCTGA AGGCTGAGAG CTATGCTATG CCTAGTGACG TGTTTATAGT 2799

AAATGTCCAT TACATTGACC AAGAACGACA AGAACCATAA GCTTGCTGAG GATAGATGGG 2859

GGCGCGTCCG CGAACGACTT G 2880

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 519 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Leu Gln Arg Phe Ser Phe Phe Val Thr Leu Ala Leu Val Ala  
 1 5 10 15

Arg Ser Leu Ala Ala Ile Gly Pro Val Ala Ser Leu Val Val Ala Asn  
 20 25 30

Ala Pro Val Ser Pro Asp Gly Phe Leu Arg Asp Ala Ile Val Val Asn  
 35 40 45

Gly Val Val Pro Ser Pro Leu Ile Thr Gly Lys Lys Gly Asp Arg Phe  
 50 55 60

Gln Leu Asn Val Val Asp Thr Leu Thr Asn His Ser Met Leu Lys Ser  
 65 70 75 80

Thr Ser Ile His Trp His Gly Phe Phe Gln Ala Gly Thr Asn Trp Ala  
 85 90 95

Glu Gly Pro Ala Phe Val Asn Gln Cys Pro Ile Ala Ser Gly His Ser  
 100 105 110

Phe Leu Tyr Asp Phe His Val Pro Asp Gln Ala Gly Thr Phe Trp Tyr  
 115 120 125

His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Phe  
 130 135 140  
 Val Val Tyr Asp Pro Lys Asp Pro His Ala Ser Arg Tyr Asp Val Asp  
 145 150 155 160  
 Asn Glu Ser Thr Val Ile Thr Leu Thr Asp Trp Tyr His Thr Ala Ala  
 165 170 175  
 Arg Leu Gly Pro Lys Phe Pro Leu Gly Ala Asp Ala Thr Leu Ile Asn  
 180 185 190  
 Gly Leu Gly Arg Ser Ala Ser Thr Pro Thr Ala Ala Leu Ala Val Ile  
 195 200 205  
 Asn Val Gln His Gly Lys Arg Tyr Arg Phe Arg Leu Val Ser Ile Ser  
 210 215 220  
 Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Leu Thr Val  
 225 230 235 240  
 Ile Glu Val Asp Gly Ile Asn Ser Gln Pro Leu Leu Val Asp Ser Ile  
 245 250 255  
 Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val Leu Asn Ala Asn Gln  
 260 265 270  
 Thr Val Gly Asn Tyr Trp Val Arg Ala Asn Pro Asn Phe Gly Thr Val  
 275 280 285  
 Gly Phe Ala Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr Gln Gly Ala  
 290 295 300  
 Pro Val Ala Glu Pro Thr Thr Thr Gln Thr Pro Ser Val Ile Pro Leu  
 305 310 315 320  
 Ile Glu Thr Asn Leu His Pro Leu Ala Arg Met Pro Val Pro Gly Ser  
 325 330 335  
 Pro Thr Pro Gly Gly Val Asp Lys Ala Leu Asn Leu Ala Phe Asn Phe  
 340 345 350  
 Asn Gly Thr Asn Phe Phe Ile Asn Asn Ala Thr Phe Thr Pro Pro Thr  
 355 360 365  
 Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala Gln Thr Ala Gln Asp  
 370 375 380  
 Leu Leu Pro Ala Gly Ser Val Tyr Pro Leu Pro Ala His Ser Thr Ile  
 385 390 395 400  
 Glu Ile Thr Leu Pro Ala Thr Ala Leu Ala Pro Gly Ala Pro His Pro  
 405 410 415  
 Phe His Leu His Gly His Ala Phe Ala Val Val Arg Ser Ala Gly Ser  
 420 425 430  
 Thr Thr Tyr Asn Tyr Asn Asp Pro Ile Phe Arg Asp Val Val Ser Thr  
 435 440 445  
 Gly Thr Pro Ala Ala Gly Asp Asn Val Thr Ile Arg Phe Gln Thr Asp  
 450 455 460  
 Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Asp  
 465 470 475 480  
 Ala Gly Phe Ala Ile Val Phe Ala Glu Asp Val Ala Asp Val Lys Ala  
 485 490 495



Ala Asn Pro Val Pro Lys Ala Trp Ser Asp Leu Cys Pro Ile Tyr Asp  
 500 505 510

Gly Leu Ser Glu Ala Asn Gln  
 515

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 3102 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Polyporus pinsitus
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 666..720
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 790..845
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1125..1182
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1390..1450
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1607..1661
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1863..1918
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1976..2025
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 2227..2285
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 2403..2458
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 2576..2627
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: join (665..721, 789..846, 1124..1183, 1389..1451, 1606..1662, 1862..1919, 1975..2026, 2226..2286, 2402..2459, 2575..2628).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTTCCCAGCT AAACCAATCT CAGNCCGCTT CCTCCTAGGG AACCGAGCGA TGTGGCGGCC

60

CTCTCTATCC AAGCTGTCCA TAAGAAGACG TTCAAATGCC GCAGCAAGCG AGGAAATAAG	120
CATCTAACAG TGTTTTTCCC ATAGTCGCAT TTGCGCCGCC TGTCGGACCG ACGCCCCTAG	180
AGCGCTTTGG GAAACGTCGC AAGTGGCGGG TGTTATTCGT GTAGACGAGA CGGTATTTGT	240
CTCATCATTC CCGTGCTTCA GGTGACACA GCCCAAAGGT CTATGTACGG CCCTTCACAT	300
TCCCTGACAC ATTGACGCAA CCCTCGGTGC GCCTCCGACA GTGCCTCGGT TGTAGTATCG	360
GGACGCCCTA GGATGCAAGA TTGGAAGTCA CCAAGGCCCG AAGGGTATAA AATACCGAGA	420
GGTCTACCA CTCTGCATC TCCAGTCGCA GAGTTCCTCT CCCTTGCCAG CCACAGCTCG	480
AG ATG TCC TTC TCT AGC CTT CGC CGT GCC TTG GTC TTC CTG GGT GCT	527
Met Ser Phe Ser Ser Leu Arg Arg Ala Leu Val Phe Leu Gly Ala	
1 5 10 15	
TGC AGC AGT GCG CTG GCC TCC ATC GGC CCA GTC ACT GAG CTC GAC ATC	575
Cys Ser Ser Ala Leu Ala Ser Ile Gly Pro Val Thr Glu Leu Asp Ile	
20 25 30	
GTT AAC AAG GTC ATC GCC CCG GAT GGC GTC GCT CGT GAT ACA GTC CTC	623
Val Asn Lys Val Ile Ala Pro Asp Gly Val Ala Arg Asp Thr Val Leu	
35 40 45	
GCC GGG GGC ACG TTC CCG GGC CCA CTC ATC ACA GGA AAG AAG	665
Ala Gly Gly Thr Phe Pro Gly Pro Leu Ile Thr Gly Lys Lys	
50 55 60	
GTATGCTAAG TAGTCCCGCC CCCATCATCC TGTGGCTGAC GTTCGACGCC GCCAG	720
GGT GAC AAC TTC CGC ATC AAC GTC GTC GAC AAG TTG GTT AAC CAG ACT	768
Gly Asp Asn Phe Arg Ile Asn Val Val Asp Lys Leu Val Asn Gln Thr	
65 70 75	
ATG CTG ACA TCC ACC ACC ATT GTATGTCACT AGCTCTCGCT ATCTCGAGAC	819
Met Leu Thr Ser Thr Thr Ile	
80	
CCGCTGACCG ACAACATTTG CCGTAG CAC TGG CAC GGG ATG TTC CAG CAT	859
His Trp His Gly Met Phe Gln His	
85 90	
ACG ACG AAC TGG GCG GAT GGT CCC GCC TTT GTG ACT CAA TGC CCT ATC	917
Thr Thr Asn Trp Ala Asp Gly Pro Ala Phe Val Thr Gln Cys Pro Ile	
95 100 105	
ACC ACT GGT GAT GAT TTC CTG TAC AAC TTC CGC GTG CCC GAC CAG ACA	965
Thr Thr Gly Asp Asp Phe Leu Tyr Asn Phe Arg Val Pro Asp Gln Thr	
110 115 120	
GTACGCAAAG GGCAGCATGC GTACTCAAAG ACATCTCTAA GCATTTGCTA CCTAG	1020
GGA ACG TAC TGG TAC CAT AGC CAT CTG GCC TTG CAG TAC TGT GAT GGG	1068
Gly Thr Tyr Trp Tyr His Ser His Leu Ala Leu Gln Tyr Cys Asp Gly	
125 130 135 140	
CTT CGC GGC CCC CTG GTG ATT TAC GAT CCC CAT GAT CCG CAG GCA TAC	1116
Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro His Asp Pro Gln Ala Tyr	
145 150 155	
CTG TAT GAC GTC GAT GAC GTACGCAGCA CAGTTTCCCT AAAACGGTTA	1164
Leu Tyr Asp Val Asp Asp	
160	
ACTTCTAATT CTGTAAATAT CTTTCATAG GAG AGC ACC GTT ATC ACT CTG	1213
Glu Ser Thr Val Ile Thr Leu	
165	

GCA GAC TGG TAC CAT ACC CCG GCG CCT CTG CTG CCG CCT GCC GCG Ala Asp Trp Tyr His Thr Pro Ala Pro Leu Leu Pro Pro Ala Ala 170 175 180	1258
GTACGCCTCC ACACATCTGC ACAGCGTTCC GTATCTCATA CCCTTAAAGT TTATCGGACA	1318
ACT TTG ATT AAT GGC CTG GGT CGC TGG CCT GGC AAC CCC ACC GCC GAC Thr Leu Ile Asn Gly Leu Gly Arg Trp Pro Gly Asn Pro Thr Ala Asp 185 190 195 200	1366
CTA GCC GTC ATC GAA GTC CAG CAC GGA AAG CGC GTATGTCATA GCTCGGTTAT Leu Ala Val Ile Glu Val Gln His Gly Lys Arg 205 210	1419
CTATTCATAC TCGCGGCTC GAAGCTAAAA CCTTGTTCCA G TAC CGG TTC CGA Tyr Arg Phe Arg 215	1472
CTG GTC AGC ACC TCA TGC GAC CCC AAC TAC AAC TTC ACT ATC GAT GGC Leu Val Ser Thr Ser Cys Asp Pro Asn Tyr Asn Phe Thr Ile Asp Gly 220 225 230	1520
CAC ACC ATG ACA ATC ATC GAG GCG GAT GGG CAG AAC ACC CAG CCA CAC His Thr Met Thr Ile Ile Glu Ala Asp Gly Gln Asn Thr Gln Pro His 235 240 245	1568
CAA GTC GAC GGA CTT CAG ATC TTC GCG GCA CAG CGG TAC TCC TTC GTT Gln Val Asp Gly Leu Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val 250 255 260	1616
GTATGTTTTTC CGCATTTCGG GAAAAGGAAT TGCCTGACA GCTCGAGTGT GCGTAG	1672
CTT AAC GCT AAC CAA GCG GTC AAC AAC TAC TGG ATC CGT GCG AAC CCT Leu Asn Ala Asn Gln Ala Val Asn Asn Tyr Trp Ile Arg Ala Asn Pro 265 270 275	1720
AAC CGT GCT AAC ACT ACG GGC TTC GCC AAC GGC ATC AAC TCC GCC ATC Asn Arg Ala Asn Thr Thr Gly Phe Ala Asn Gly Ile Asn Ser Ala Ile 280 285 290 295	1768
CTG CGC TAC AAG GGG GCG CCG ATT AAG GAG CCT ACG ACG AAC CAG ACT Leu Arg Tyr Lys Gly Ala Pro Ile Lys Glu Pro Thr Thr Asn Gln Thr 300 305 310	1816
ACC ATC CGG AAC TTT TTG TGG GAG ACG GAC TTG CAC CCG CTC ACT GAC Thr Ile Arg Asn Phe Leu Trp Glu Thr Asp Leu His Pro Leu Thr Asp 315 320 325	1864
CCA CGT GCA GTAAGTTCTA CACAGTCACC AACGGTGAGC TGTGTCTGA Pro Arg Ala 330	1913
TTGCACTGTG TTATAG CCT GGC CTT CCT TTC AAG GGG GGC GTT GAC CAC Pro Gly Leu Pro Phe Lys Gly Gly Val Asp His 335 340	1962
GCT TTG AAC CTC AAC CTC ACT TTC GTACGTAGCG CCTCAGATAT CGAGTAGTCT Ala Leu Asn Leu Asn Leu Thr Phe 345	2016
ATCTCCTGAC CGATTGACAG AAT GGA TCG GAG TTC TTC ATC AAC GAT GCG Asn Gly Ser Glu Phe Phe Ile Asn Asp Ala 350 355	2066
CCT TTC GTC CCT CCG ACT GTC CCG GTG CTA CTG CAG ATC CTG AAC GGA Pro Phe Val Pro Pro Thr Val Pro Val Leu Leu Gln Ile Leu Asn Gly 360 365 370 375	2114

ACG CTC GAC GCG AAC GAC CTC CTG CCG CCC GGC AGC GTC TAC AAC CTT Thr Leu Asp Ala Asn Asp Leu Leu Pro Pro Gly Ser Val Tyr Asn Leu 380 385 390	2162
CCT CCG GAC TCC ACC ATC GAG CTG TCC ATT CCC GGA GGT GTG ACG GGT Pro Pro Asp Ser Thr Ile Glu Leu Ser Ile Pro Gly Gly Val Thr Gly 395 400 405	2210
GGC CCG CAC CCA TTC CAT TTG CAC GGG GTAATAATCT CTCTTTATAC Gly Pro His Pro Phe His Leu His Gly 410 415	2257
TTTGGTCTCC CGATGCTGAC TTTCAGTCT CATCTTCAG CAC GCT TTC TCC GTC His Ala Phe Ser Val 420	2311
GTG CGT AGC GCC GGC AGC ACC GAA TAC AAC TAC GCG AAC CCG GTG AAG Val Arg Ser Ala Gly Ser Thr Glu Tyr Asn Tyr Ala Asn Pro Val Lys 425 430 435	2359
CGC GAC ACG GTC AGC ATT GGT CTT GCG GGC GAC AAC GTC ACC GTG CGC Arg Asp Thr Val Ser Ile Gly Leu Ala Gly Asp Asn Val Thr Val Arg 440 445 450	2407
TTC GTG GTATGTTTTA CAGCCTCTCT ATCTCCGTGG GCGTTCGGAA GTTGACTGGG Phe Val 455	2463
GCGTAG ACC GAC AAC CCC GGC CCG TGG TTC CTC CAC TGT CAC ATC GAC Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp 460 465	2511
TTC CAT TTG CAA GCA GGC CTC GCC ATC GTG TTC GCG GAG GAC GCG CAG Phe His Leu Gln Ala Gly Leu Ala Ile Val Phe Ala Glu Asp Ala Gln 470 475 480 485	2559
GAC ACG AAG CTT GTG AAC CCC GTC CCT GTACGTCTTC TGGATGCATG Asp Thr Lys Leu Val Asn Pro Val Pro 490	2606
CGCTCCGCAC AGTGACTCAT CTTTGTCAAC AG GAG GAC TGG AAC AAG CTG TGC Glu Asp Trp Asn Lys Leu Cys 495 500	2659
CCC ACC TTC GAT AAG GCG ATG AAC ATC ACG GTT TGAGCGATGC Pro Thr Phe Asp Lys Ala Met Asn Ile Thr Val 505 510	2702
GTGGCGCTCA TGGTCATTTT CTTGGAATCT TTGCATAGGG CTGCAGCACG CTGGATACTC	2762
TTTCCCTTAG CAGGATATTA TTTAATGACC CCTGCGTTTA GTGCTTAGTT AGCTTTACTA	2822
CTGGTTGTAA TGTACGACG ATGCGTAATT CGGATAATGC TATCAATGTG TATATTATGA	2882
CACGCGTCAT GCGCGATGCT TGAGTTGCAA GGTGCGTTTC CGATGCTCGA CATAAACGTT	2942
TCACTTACAT ACACATTGGG TCTAGAACTG GATCTATCCA TGTATACAAA AACTCCTCAT	3002
ACAGCTGACT GGGGCGCTCT AGAGCATGGG TCCGATTGAT CAGATGTCGC GAACACGAGC	3062
CTCCTGAGCT CGAGGACTCT GAGAAGCGGC GGTGCGTTCT	3102

## (2) INFORMATION FOR SEQ ID NO: 6

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 512 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Polyporus pinsitus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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Met Ser Phe Ser Ser Leu Arg Arg Ala Leu Val Phe Leu Gly Ala Cys
1      5      10      15
Ser Ser Ala Leu Ala Ser Ile Gly Pro Val Thr Glu Leu Asp Ile Val
20     25     30
Asn Lys Val Ile Ala Pro Asp Gly Val Ala Arg Asp Thr Val Leu Ala
35     40     45
Gly Gly Thr Phe Pro Gly Pro Leu Ile Thr Gly Lys Lys Gly Asp Asn
50     55     60
Phe Arg Ile Asn Val Val Asp Lys Leu Val Asn Gln Thr Met Leu Thr
65     70     75     80
Ser Thr Thr Ile His Trp His Gly Met Phe Gln His Thr Thr Asn Trp
85     90     95
Ala Asp Gly Pro Ala Phe Val Thr Gln Cys Pro Ile Thr Thr Gly Asp
100    105    110
Asp Phe Leu Tyr Asn Phe Arg Val Pro Asp Gln Thr Gly Thr Tyr Trp
115    120    125
Tyr His Ser His Leu Ala Leu Gln Tyr Cys Asp Gly Leu Arg Gly Pro
130    135    140
Leu Val Ile Tyr Asp Pro His Asp Pro Gln Ala Tyr Leu Tyr Asp Val
145    150    155    160
Asp Asp Glu Ser Thr Val Ile Thr Leu Ala Asp Trp Tyr His Thr Pro
165    170    175
Ala Pro Leu Leu Pro Pro Ala Ala Thr Leu Ile Asn Gly Leu Gly Arg
180    185    190
Trp Pro Gly Asn Pro Thr Ala Asp Leu Ala Val Ile Glu Val Gln His
195    200    205
Gly Lys Arg Tyr Arg Phe Arg Leu Val Ser Thr Ser Cys Asp Pro Asn
210    215    220
Tyr Asn Phe Thr Ile Asp Gly His Thr Met Thr Ile Ile Glu Ala Asp
225    230    235    240
Gly Gln Asn Thr Gln Pro His Gln Val Asp Gly Leu Gln Ile Phe Ala
245    250    255
Ala Gln Arg Tyr Ser Phe Val Leu Asn Ala Asn Gln Ala Val Asn Asn
260    265    270
Tyr Trp Ile Arg Ala Asn Pro Asn Arg Ala Asn Thr Thr Gly Phe Ala
275    280    285
Asn Gly Ile Asn Ser Ala Ile Leu Arg Tyr Lys Gly Ala Pro Ile Lys
290    295    300
Glu Pro Thr Thr Asn Gln Thr Thr Ile Arg Asn Phe Leu Trp Glu Thr
305    310    315    320

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Asp Leu His Pro Leu Thr Asp Pro Arg Ala Pro Gly Leu Pro Phe Lys
      325                      330                      335
Gly Gly Val Asp His Ala Leu Asn Leu Asn Leu Thr Phe Asn Gly Ser
      340                      345                      350
Glu Phe Phe Ile Asn Asp Ala Pro Phe Val Pro Pro Thr Val Pro Val
      355                      360                      365
Leu Leu Gln Ile Leu Asn Gly Thr Leu Asp Ala Asn Asp Leu Leu Pro
      370                      375                      380
Pro Gly Ser Val Tyr Asn Leu Pro Pro Asp Ser Thr Ile Glu Leu Ser
      385                      390                      395                      400
Ile Pro Gly Gly Val Thr Gly Gly Pro His Pro Phe His Leu His Gly
      405                      410                      415
His Ala Phe Ser Val Val Arg Ser Ala Gly Ser Thr Glu Tyr Asn Tyr
      420                      425                      430
Ala Asn Pro Val Lys Arg Asp Thr Val Ser Ile Gly Leu Ala Gly Asp
      435                      440                      445
Asn Val Thr Val Arg Phe Val Thr Asp Asn Pro Gly Pro Trp Phe Leu
      450                      455                      460
His Cys His Ile Asp Phe His Leu Gln Ala Gly Leu Ala Ile Val Phe
      465                      470                      475                      480
Ala Glu Asp Ala Gln Asp Thr Lys Leu Val Asn Pro Val Pro Glu Asp
      485                      490                      495
Trp Asn Lys Leu Cys Pro Thr Phe Asp Lys Ala Met Asn Ile Thr Val
      500                      505                      510

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## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2860 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 851..905
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1266..1320
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1351..1376
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1416..1468
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1625..1683
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1882..1934

- (ix) FEATURE:  
 (A) NAME/KEY: intron  
 (B) LOCATION: 2202..2252
- (ix) FEATURE:  
 (A) NAME/KEY: intron  
 (B) LOCATION: 2370..2425
- (ix) FEATURE:  
 (A) NAME/KEY: intron  
 (B) LOCATION: 2543..2599
- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: join(540..725, 782..850, 906..1025, 1086..1265,  
 1321..1350, 1377..1415, 1469..1624, 1684..1881,  
 1935..2201, 2253..2369, 2426..2542, 2600..2653)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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GGGGGGCGCG TCAATGGTCC GTTTGCGAAC ACATATGCAG GATAAACAGT GCGAAATATC      60
AATGTGGCGG CGACACAACC TCGCCGGCCG ACACTCGACG CTGTTGATCA TGATCATGTC      120
TTGTGAGCAT TCTATACGCA GCCTTGGAAA TCTCAGGCGA ATTTGTCTGA ATTGCGCTGG      180
GAGGCTGGCA GCGCAGATCG GTGTGTCGGT GCAGTAGCCG ACGCAGCACC TGGCGGAAGC      240
CGACATCTCG GGTACGACTT GATCTCCGCC AGATCACTGC GGTTCGCCCA TCGGCCGCGG      300
GGCCCATTTCT GTGTGTGCGC TGTAGCACTC TGCATTTCAGG CTCAACGTAT CCATGCTAGA      360
GGACCGTCCA GCTGTTGGCG CACGATTCGC GCAGAAAGCT GTACAGGCAG ATATAAGGAT      420
GTCCGTCGGT CAGAGACTCG TCACTCACAA GCCTCTTTTC CTCTTCGCCT TTCCAGCCTC      480
TTCCAACGCC TGCCATCGTC CTCTTAGTTC GTCGTCCAT TCTTTCTGCG TAGTTAATC      539
ATG GGC AGG TTC TCA TCT CTC TGC GCG CTC ACC GCC GTC ATC CAC TCT      587
Met Gly Arg Phe Ser Ser Leu Cys Ala Leu Thr Ala Val Ile His Ser
  1             5             10             15

TTT GGT CGT GTC TCC GCC GCT ATC GGG CCT GTG ACC GAC CTC ACC ATC      635
Phe Gly Arg Val Ser Ala Ala Ile Gly Pro Val Thr Asp Leu Thr Ile
  20             25             30

TCC AAT GGG GAC GTT TCT CCC GAC GGC TTC ACT CGT GCC GCA GTG CTT      683
Ser Asn Gly Asp Val Ser Pro Asp Gly Phe Thr Arg Ala Ala Val Leu
  35             40             45

GCA AAC GGC GTC TTC CCG GGT CCT CTT ATC ACG GGA AAC AAG      725
Ala Asn Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys
  50             55             60

GTACGTGGCA TCGGTTTCAGT CTACACCCTA CAAGCCTTCT AACTCTTTTA CCACAG      781
GGC GAC AAC TTC CAG ATC AAT GTT ATC GAC AAC CTC TCT AAC GAG ACG      829
Gly Asp Asn Phe Gln Ile Asn Val Ile Asp Asn Leu Ser Asn Glu Thr
  65             70             75

ATG TTG AAG TCG ACC TCC ATC GTATGTGCTT CTA CTGCTTC TTAGTCTTGG      880
Met Leu Lys Ser Thr Ser Ile
  80             85

CAATGGCTCA AGGTCTCCTC CGCAG CAT TGG CAC GGC TTC TTC CAG AAG GGT      932
His Trp His Gly Phe Phe Gln Lys Gly
  90

ACT AAC TGG GCT GAT GGA GCT GCC TTC GTC AAC CAG TGC CCT ATC GCG      980

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Thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala 95 100 105 110	
ACG GGG AAC TCT TTC CTT TAC GAC TTC ACC GCG ACG GAC CAA GCA Thr Gly Asn Ser Phe Leu Tyr Asp Phe Thr Ala Thr Asp Gln Ala 115 120 125	1025
GTCAGTGCCT GTGGCGCTTA TGTTTTCCCG TAATCAGCAG CTAACACTCC GCACCCACAG	1085
GGC ACC TTC TGG TAC CAC AGT CAC TTG TCT ACG CAG TAC TGC GAT GGT Gly Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly 130 135 140	1133
TTG CGG GGC CCG ATG GTC GTA TAC GAC CCG AGT GAC CCG CAT GCG GAC Leu Arg Gly Pro Met Val Val Tyr Asp Pro Ser Asp Pro His Ala Asp 145 150 155	1181
CTT TAC GAC GTC GAC GAC GAG ACC ACG ATC ATC ACG CTC TCT GAT TGG Leu Tyr Asp Val Asp Asp Glu Thr Thr Ile Ile Thr Leu Ser Asp Trp 160 165 170	1229
TAT CAC ACC GCT GCT TCG CTC GGT GCT GCC TTC CCG GTAAGTTTAC Tyr His Thr Ala Ala Ser Leu Gly Ala Ala Phe Pro 175 180 185	1275
CCCAGCGCAC GGAGTTAAGA CCGGATCTAA CTGTAATACG TTCAG ATT GGC TCG Ile Gly Ser	1329
GAC TCT ACC CTG ATT AAC GGC GTTGGCCGCT TCGCGGGTGG TGACAG ACT GAC Asp Ser Thr Leu Ile Asn Gly Thr Asp 190 195	1382
CTT GCG GTT ATC ACT GTC GAG CAG GGC AAG CGC GTTAGTGATA CCCTCTACAG Leu Ala Val Ile Thr Val Glu Gln Gly Lys Arg 200 205	1435
TTGACACTGT GCCATTGCTG ACAGTACTCT CAG TAC CGT ATG CGT CTT CTC TCG Tyr Arg Met Arg Leu Leu Ser 210 215	1489
CTG TCT TGC GAC CCC AAC TAT GTC TTC TCC ATT GAC GGC CAC AAC ATG Leu Ser Cys Asp Pro Asn Tyr Val Phe Ser Ile Asp Gly His Asn Met 220 225 230	1537
ACC ATC ATC GAG GCC GAC GCC GTC AAC CAC GAG CCC CTC ACG GTT GAC Thr Ile Ile Glu Ala Asp Ala Val Asn His Glu Pro Leu Thr Val Asp 235 240 245	1585
TCC ATC CAG ATC TAC GCC GGC CAA CGT TAC TCC TTC GTC GTACGTATTC Ser Ile Gln Ile Tyr Ala Gly Gln Arg Tyr Ser Phe Val 250 255 260	1634
CGAACAGCCA TGATCACGCC AAGCCCAGTG CTAACGCGCC TACCCCTCAG CTT ACC Leu Thr	1689
GCT GAC CAG GAC ATC GAC AAC TAC TTC ATC CGT GCC CTG CCC AGC GCC Ala Asp Gln Asp Ile Asp Asn Tyr Phe Ile Arg Ala Leu Pro Ser Ala 265 270 275	1737
GGT ACC ACC TCG TTC GAC GGC GGC ATC AAC TCG GCT ATC CTG CGC TAC Gly Thr Thr Ser Phe Asp Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr 280 285 290	1785
TCT GGT GCC TCC GAG GTT GAC CCG ACG ACC ACG GAG ACC ACG AGC GTC Ser Gly Ala Ser Glu Val Asp Pro Thr Thr Glu Thr Thr Ser Val 295 300 305 310	1833



CTC CCC CTC GAC GAG GCG AAC CTC GTG CCC CTT GAC AGC CCC GCT GCT Leu Pro Leu Asp Glu Ala Asn Leu Val Pro Leu Asp Ser Pro Ala Ala 315 320 325	1881
GTACGTCGTA TTCTGCGCTT GCAAGGATCG CACATACTAA CATGCTCTTG TAG CCC Pro	1937
GGT GAC CCC AAC ATT GGC GGT GTC GAC TAC GCG CTG AAC TTG GAC TTC Gly Asp Pro Asn Ile Gly Gly Val Asp Tyr Ala Leu Asn Leu Asp Phe 330 335 340	1985
AAC TTC GAT GGC ACC AAC TTC TTC ATC AAC GAC GTC TCC TTC GTG TCC Asn Phe Asp Gly Thr Asn Phe Ile Asn Asp Val Ser Phe Val Ser 345 350 355	2033
CCC ACG GTC CCT GTC CTC CTC CAG ATT CTT AGC GGC ACC ACC TCC GCG Pro Thr Val Pro Val Leu Leu Gln Ile Leu Ser Gly Thr Thr Ser Ala 360 365 370 375	2081
GCC GAC CTT CTC CCC AGC GGT AGT CTC TTC GCG GTC CCG TCC AAC TCG Ala Asp Leu Leu Pro Ser Gly Ser Leu Phe Ala Val Pro Ser Asn Ser 380 385 390	2129
ACG ATC GAG ATC TCG TTC CCC ATC ACC GCG ACG AAC GCT CCC GGC GCG Thr Ile Glu Ile Ser Phe Pro Ile Thr Ala Thr Asn Ala Pro Gly Ala 395 400 405	2177
CCG CAT CCC TTC CAC TTG CAC GGT GTACGTGTCC CATCTCATAT GCTACGGAGC Pro His Pro Phe His Leu His Gly 410 415	2231
TCCACGCTGA CCGCCCTATA G CAC ACC TTC TCT ATC GTT CGT ACC GCC GGC His Thr Phe Ser Ile Val Arg Thr Ala Gly 420 425	2282
AGC ACG GAT ACG AAC TTC GTC AAC CCC GTC CGC CGC GAC GTC GTG AAC Ser Thr Asp Thr Asn Phe Val Asn Pro Val Arg Arg Asp Val Val Asn 430 435 440	2330
ACC GGT ACC GTC GGC GAC AAC GTC ACC ATC CGC TTC ACG GTACGCAGCA Thr Gly Thr Val Gly Asp Asn Val Thr Ile Arg Phe Thr 445 450	2379
CTCTCCTAAC ATTCCCACTG CGCGATCACT GACTCCTCGC CCACAG ACT GAC AAC Thr Asp Asn 455	2434
CCC GGC CCC TGG TTC CTC CAC TGC CAC ATC GAC TTC CAC TTG GAG GCC Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Glu Ala 460 465 470	2482
GGT TTC GCC ATC GTC TTC AGC GAG GAC ACC GCC GAC GTC TCG AAC ACG Gly Phe Ala Ile Val Phe Ser Glu Asp Thr Ala Asp Val Ser Asn Thr 475 480 485	2530
ACC ACG CCC TCG GTACGTTGTG CTCCCGTGCC CATCTCCGCG CGCCTGACTA Thr Thr Pro Ser 490	2582
ACGAGCACCC CTTACAG ACT GCT TGG GAA GAT CTG TGC CCC ACG TAC AAC Thr Ala Trp Glu Asp Leu Cys Pro Thr Tyr Asn 495 500	2632
GCT CTT GAC TCA TCC GAC CTC TAATCGGTTT AAAGGGTCGC TCGCTACCTT Ala Leu Asp Ser Ser Asp Leu 505 510	2683

AGTAGGTAGA CTTATGCACC GGACATTATC TACAATGGAC TTAAATTGG GTTAACGGCC 2743  
 GTTATACATA CGCGCACGTA GTATAAGGT TCTCTGGATT GGTCGGACCT ACAGACTGCA 2803  
 ATTTTCGTGA CCTATCAACT GTATATTGAA GCACGACAGT GAATGGAAAT AGAGACA 2860

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 511 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Gly Arg Phe Ser Ser Leu Cys Ala Leu Thr Ala Val Ile His Ser  
 1 5 10 15  
 Phe Gly Arg Val Ser Ala Ala Ile Gly Pro Val Thr Asp Leu Thr Ile  
 20 25 30  
 Ser Asn Gly Asp Val Ser Pro Asp Gly Phe Thr Arg Ala Ala Val Leu  
 35 40 45  
 Ala Asn Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys Gly Asp  
 50 55 60  
 Asn Phe Gln Ile Asn Val Ile Asp Asn Leu Ser Asn Glu Thr Met Leu  
 65 70 75 80  
 Lys Ser Thr Ser Ile His Trp His Gly Phe Phe Gln Lys Gly Thr Asn  
 85 90 95  
 Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala Thr Gly  
 100 105 110  
 Asn Ser Phe Leu Tyr Asp Phe Thr Ala Thr Asp Gln Ala Gly Thr Phe  
 115 120 125  
 Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly  
 130 135 140  
 Pro Met Val Val Tyr Asp Pro Ser Asp Pro His Ala Asp Leu Tyr Asp  
 145 150 155 160  
 Val Asp Asp Glu Thr Thr Ile Ile Thr Leu Ser Asp Trp Tyr His Thr  
 165 170 175  
 Ala Ala Ser Leu Gly Ala Ala Phe Pro Ile Gly Ser Asp Ser Thr Leu  
 180 185 190  
 Ile Asn Gly Thr Asp Leu Ala Val Ile Thr Val Glu Gln Gly Lys Arg  
 195 200 205  
 Tyr Arg Met Arg Leu Leu Ser Leu Ser Cys Asp Pro Asn Tyr Val Phe  
 210 215 220  
 Ser Ile Asp Gly His Asn Met Thr Ile Ile Glu Ala Asp Ala Val Asn  
 225 230 235 240  
 His Glu Pro Leu Thr Val Asp Ser Ile Gln Ile Tyr Ala Gly Gln Arg  
 245 250 255  
 Tyr Ser Phe Val Leu Thr Ala Asp Gln Asp Ile Asp Asn Tyr Phe Ile  
 260 265 270

Arg Ala Leu Pro Ser Ala Gly Thr Thr Ser Phe Asp Gly Gly Ile Asn  
 275 280 285  
 Ser Ala Ile Leu Arg Tyr Ser Gly Ala Ser Glu Val Asp Pro Thr Thr  
 290 295 300  
 Thr Glu Thr Thr Ser Val Leu Pro Leu Asp Glu Ala Asn Leu Val Pro  
 305 310 315 320  
 Leu Asp Ser Pro Ala Ala Pro Gly Asp Pro Asn Ile Gly Gly Val Asp  
 325 330 335  
 Tyr Ala Leu Asn Leu Asp Phe Asn Phe Asp Gly Thr Asn Phe Phe Ile  
 340 345 350  
 Asn Asp Val Ser Phe Val Ser Pro Thr Val Pro Val Leu Leu Gln Ile  
 355 360 365  
 Leu Ser Gly Thr Thr Ser Ala Ala Asp Leu Leu Pro Ser Gly Ser Leu  
 370 375 380  
 Phe Ala Val Pro Ser Asn Ser Thr Ile Glu Ile Ser Phe Pro Ile Thr  
 385 390 395 400  
 Ala Thr Asn Ala Pro Gly Ala Pro His Pro Phe His Leu His Gly His  
 405 410 415  
 Thr Phe Ser Ile Val Arg Thr Ala Gly Ser Thr Asp Thr Asn Phe Val  
 420 425 430  
 Asn Pro Val Arg Arg Asp Val Val Asn Thr Gly Thr Val Gly Asp Asn  
 435 440 445  
 Val Thr Ile Arg Phe Thr Thr Asp Asn Pro Gly Pro Trp Phe Leu His  
 450 455 460  
 Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val Phe Ser  
 465 470 475 480  
 Glu Asp Thr Ala Asp Val Ser Asn Thr Thr Thr Pro Ser Thr Ala Trp  
 485 490 495  
 Glu Asp Leu Cys Pro Thr Tyr Asn Ala Leu Asp Ser Ser Asp Leu  
 500 505 510

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2925 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Polyporus pinsitus*

## (ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 734..808

## (ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 878..932

## (ix) FEATURE:

- (A) NAME/KEY: intron

(B) LOCATION: 1051..1104

(ix) FEATURE:

(A) NAME/KEY: intron  
(B) LOCATION: 1219..1270

(ix) FEATURE:

(A) NAME/KEY: intron  
(B) LOCATION: 1336..1397

(ix) FEATURE:

(A) NAME/KEY: intron  
(B) LOCATION: 1713..7744

(ix) FEATURE:

(A) NAME/KEY: intron  
(B) LOCATION: 2030..2085

(ix) FEATURE:

(A) NAME/KEY: intron  
(B) LOCATION: 2308..2375

(ix) FEATURE:

(A) NAME/KEY: intron  
(B) LOCATION: 2492..2569

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: join (733..809, 877..933, 1050..1105, 1218..1271,  
1335..1398, 1712..1775, 2029..2086, 2307..2376, 2492..2570).  
2542..2600).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTCATAACTC TTCGCTTCTA GCATGGGGGC TGCACACACC TGACAGACCC TTCGGGAGGC	60
GAAGTCGAAT GCAGCGTACT CTATCNCACC TCCAGGAAAG GTAGGGATGG ACNCCGTGCA	120
CCAACAAC TG TCTCTCCACC AGCAACCATC CCTTGGATAT GTCTCCACAC ACCCGGTGTC	180
TACAAGCGGG GATCTGTGCT GGTGAAGTGC TGTCTCCGGA GCGGCGGCGG CGAGCGACCA	240
GAACCCGAAC CAGTGCTAGT GCGCGACACC CGCGAGACAA TTGTGCAGGG TGAGTTATAT	300
TCTTCGTGAG ACGGCGCTGC GCGTCGGCAC TGAAAGCGTC GCAGTTAGGT GATGCAGCGG	360
TCCGCGCTAT TTTTGACGTC TGGCAGCTAT CCTAAGCCGC GCCTCCATAC ACCCCAGGCG	420
CTCTCGTTTG CTATAGGTAT AAATCCCTCA GCTTCAGAGC GTCGATCCTC ATCCCACACG	480
ACACCCGTTT CAGTCTTCTC GTAGCGCATT CCCTAGCCGC CCAGCCTCCG CTTTCGTTTT	540
CAAC ATG GGC AAG TAT CAC TCT TTT GTG AAC GTC GTC GCC CTT AGT CTT	589
Met Gly Lys Tyr His Ser Phe Val Asn Val Val Ala Leu Ser Leu	
1 5 10 15	
TCT TTG AGC GGT CGT GTG TTC GGC GCC ATT GGG CCC GTC ACC GAC TTG	637
Ser Leu Ser Gly Arg Val Phe Gly Ala Ile Gly Pro Val Thr Asp Leu	
20 25 30	
ACT ATC TCT AAC GCC GAT GTT ACG CCT GAC GGC ATT ACT CTT GCT GCT	685
Thr Ile Ser Asn Ala Asp Val Thr Pro Asp Gly Ile Thr Arg Ala Ala	
35 40 45	
GTC CTC GCG GGC GGC GTT TTC CCC GGG CCC CTC ATT ACC GGC AAC AAG	733
Val Leu Ala Gly Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys	
50 55 60	
GTGAGCCGCG AAACCTTCTA CTAGCGCGCT CGTACGGTGC ACCGTTACTG AAGCCACACT	793

TTGCGCTGTC AACAG GGG GAT GAA TTC CAG ATC AAT GTC ATC GAC AAC CTG Gly Asp Glu Phe Gln Ile Asn Val Ile Asp Asn Leu 65 70 75	844
ACC AAC GAG ACC ATG TTG AAG TCG ACC ACA ATC GTAAGGTGCT TGCTCCCAT Thr Asn Glu Thr Met Leu Lys Ser Thr Thr Ile 80 85	897
ATTAAGCCCCG TCGCTGACTC GAAGTTTATC TGTAG CAC TGG CAT GGT ATC TTC His Trp His Gly Ile Phe 90	950
CAG GCC GGC ACC AAC TGG GCA GAC GGC GCG GCC TTC GTG AAC CAG TGC Gln Ala Gly Thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys 95 100 105	998
CCT ATC GCC ACG GGA AAC TCG TTC TTG TAC GAC TTC ACC GTT CCT GAT Pro Ile Ala Thr Gly Asn Ser Phe Leu Tyr Asp Phe Thr Val Pro Asp 110 115 120	1046
CAA GCC GTACGTTTAT ACACCTCCCT TTCTGCGGCA TACTCTGACG CGCCGCTGGA Gln Ala 125	1102
TCAG GGC ACC TTC TGG TAC CAC AGC CAC CTG TCC ACC CAG TAC TGT GAC Gly Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp 130 135 140	1151
GGC CTG CGC GGT CCT CTT GTG GTC TAC GAC CCC GAC GAT CCC AAC GCG Gly Leu Arg Gly Pro Leu Val Val Tyr Asp Pro Asp Asp Pro Asn Ala 145 150 155	1199
TCT CTT TAC GAC GTC GAT GAC GTAAGCAGGC TACTTGTGGA CTTGTATGGA Ser Leu Tyr Asp Val Asp Asp 160	1250
TGTATCTCAC GCTCCCTAC AG GAT ACT ACG GTT ATT ACG CTT GCG GAC TGG Asp Thr Thr Val Ile Thr Leu Ala Asp Trp 165 170	1302
TAC CAC ACT GCG GCG AAG CTG GGC CCT GCC TTC CCC GTGAGTCTAC Tyr His Thr Ala Ala Lys Leu Gly Pro Ala Phe Pro 175 180 185	1348
TCCTCCTCGT GTGTAAACAT AGGTGACGGC CGCTGATACG AGAGCTACCA G GCG GGT Ala Gly	1405
CCG GAT AGC GTC TTG ATC AAT GGT CTT GGT CGG TTC TCC GGC GAT GGT Pro Asp Ser Val Leu Ile Asn Gly Leu Gly Arg Phe Ser Gly Asp Gly 190 195 200	1453
GGA GGA GCG ACA AAC CTC ACC GTG ATC ACC GTC ACG CAA GGC AAA CGG Gly Gly Ala Thr Asn Leu Thr Val Ile Thr Val Thr Gln Gly Lys Arg 205 210 215 220	1501
GTGAGTCCCG CCTGAGCTGG CCTCAATAGC GATATTGACG AGTCCATGCC CTCCCAG	1558
TAC CGC TTC CGC CTT GTG TCG ATC TCG TGC GAC CCC AAC TTC ACG TTC Tyr Arg Phe Arg Leu Val Ser Ile Ser Cys Asp Pro Asn Phe Thr Phe 225 230 235	1606
TCG ATC GAC GGG CAC AAC ATG ACC ATC ATC GAG GTG GAC GGT GTC AAC Ser Ile Asp Gly His Asn Met Thr Ile Ile Glu Val Asp Gly Val Asn 240 245 250	1654
CAC GAG GCC TTG GAC GTC GAC TCC ATT CAG ATT TTT GCG GGC CAG CGG His Glu Ala Leu Asp Val Asp Ser Ile Gln Ile Phe Ala Gly Gln Arg 255 260 265	1702

TAC TCC TTC ATC GTACGTTCCC TTGCCCTCGT GCTATATCCG CCCGTCGCT Tyr Ser Phe Ile 270	1754
CACAGAGGCT TCTATATCGC AG CTC AAC GCC AAC CAG TCC ATC GAC AAC Leu Asn Ala Asn Gln Ser Ile Asp Asn 275 280	1803
TAC TGG ATC CGC GCG ATC CCC AAC ACC GGT ACC ACC GAC ACC ACG GGC Tyr Trp Ile Arg Ala Ile Pro Asn Thr Gly Thr Thr Asp Thr Thr Gly 285 290 295	1851
GGC GTG AAC TCT GCT ATT CTT CGC TAC GAC ACC GCA GAA GAT ATC GAG Gly Val Asn Ser Ala Ile Leu Arg Tyr Asp Thr Ala Glu Asp Ile Glu 300 305 310	1899
CCT ACG ACC AAC GCG ACC ACC TCC GTC ATC CCT CTC ACC GAG ACG GAT Pro Thr Thr Asn Ala Thr Thr Ser Val Ile Pro Leu Thr Glu Thr Asp 315 320 325	1947
CTG GTG CCG CTC GAC AAC CCT GCG GCT CCC GGT GAC CCC CAG GTC GGC Leu Val Pro Leu Asp Asn Pro Ala Ala Pro Gly Asp Pro Gln Val Gly 330 335 340 345	1995
GGT GTT GAC CTG GCT ATG AGT CTC GAC TTC TCC TTC GTGAGTCCCA Gly Val Asp Leu Ala Met Ser Leu Asp Phe Ser Phe 350 355	2041
CAGCACTCCG CGCCATTTTCG CTTATTTACG CAGGAGTATT GTTCAG AAC GGT TCC Asn Gly Ser 360	2096
AAC TTC TTT ATC AAC AAC GAG ACC TTC GTC CCG CCC ACA GTT CCC GTG Asn Phe Phe Ile Asn Asn Glu Thr Phe Val Pro Pro Thr Val Pro Val 365 370 375	2144
CTC CTG CAG ATT TTG AGT GGT GCG CAG GAC GCG GCG AGC CTG CTC CCC Leu Leu Gln Ile Leu Ser Gly Ala Gln Asp Ala Ala Ser Leu Leu Pro 380 385 390	2192
AAC GGG AGT GTC TAC ACA CTC CCT TCG AAC TCG ACC ATT GAG ATC TCG Asn Gly Ser Val Tyr Thr Leu Pro Ser Asn Ser Thr Ile Glu Ile Ser 395 400 405	2240
TTC CCC ATC ATC ACC ACC GAC GGT GTT CTG AAC GCG CCC GGT GCT CCG Phe Pro Ile Ile Thr Thr Asp Gly Val Leu Asn Ala Pro Gly Ala Pro 410 415 420	2288
CAC CCG TTC CAT CTC CAC GGC GTAAGTCCTT GCTTTCCTCA GTGCCTCGCT His Pro Phe His Leu His Gly 425 430	2339
TCCACGACGT CCACTGATCC CACACATCCC ATGTGCAG CAC ACC TTC TCG GTG His Thr Phe Ser Val 435	2392
GTG CGC AGC GCC GGG AGC TCG ACC TTC AAC TAC GCC AAC CCA GTC CGC Val Arg Ser Ala Gly Ser Ser Thr Phe Asn Tyr Ala Asn Pro Val Arg 440 445 450	2440
CGG GAC ACC GTC AGT ACT GGT AAC TCT GGC GAC AAC GTC ACT ATC CGC Arg Asp Thr Val Ser Thr Gly Asn Ser Gly Asp Asn Val Thr Ile Arg 455 460 465	2488
TTC ACG GTACGTCTTC TCCGGAGCCC TCCCACCCGT GTGTCCGCTG AGCGCTGAAC Phe Thr 470	2544
ACCGCCACC GTGCTGCTGC TGC GCAG ACC GAC AAC CCA GGC CCG TGG TTC	2595

Thr Asp Asn Pro Gly Pro Trp Phe  
475

CTC CAC TGC CAC ATC GAC TTC CAC CTG GAG GCC GGC TTC GCC ATC GTC 2643  
Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val  
480 485 490

TGG GGG GAG GAC ACT GCG GAC ACC GCG TCC GCG AAT CCC GTT CCT 2688  
Trp Gly Glu Asp Thr Ala Asp Thr Ala Ser Ala Asn Pro Val Pro  
495 500 505

GTACGTCGTG CCTGCTGAGC TCTTTGTGCC CGAACAGGGT GCTGATCGTG CCTTCCTCCG 2748

TGCAG ACG GCG TGG AGC GAT TTG TGC CCC ACT TAC GAT GCT TTG GAC TCG 2798  
Thr Ala Trp Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Ser  
510 515 520

TCC GAC CTC TGATCGACAA GGCATGAAGG CTGAAGCAGC TCGGGTCAAT 2847  
Ser Asp Leu  
525

TCTCGAACAC ACTTTACTCG AACATTTCATT TTTCTTTGGC TCGGGATCGG AACAAATCAT 2907

GGGGGGGCCG GACCGTCT 2925

## (2) INFORMATION FOR SEQ ID NO: 10

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 527 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Polyporus pinsitus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Gly Lys Tyr His Ser Phe Val Asn Val Val Ala Leu Ser Leu Ser  
1 5 10 15

Leu Ser Gly Arg Val Phe Gly Ala Ile Gly Pro Val Thr Asp Leu Thr  
20 25 30

Ile Ser Asn Ala Asp Val Thr Pro Asp Gly Ile Thr Arg Ala Ala Val  
35 40 45

Leu Ala Gly Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys Gly  
50 55 60

Asp Glu Phe Gln Ile Asn Val Ile Asp Asn Leu Thr Asn Glu Thr Met  
65 70 75 80

Leu Lys Ser Thr Thr Ile His Trp His Gly Ile Phe Gln Ala Gly Thr  
85 90 95

Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala Thr  
100 105 110

Gly Asn Ser Phe Leu Tyr Asp Phe Thr Val Pro Asp Gln Ala Gly Thr  
115 120 125

Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg  
130 135 140

Gly Pro Leu Val Val Tyr Asp Pro Asp Asp Pro Asn Ala Ser Leu Tyr  
145 150 155 160

Asp Val Asp Asp Asp Thr Thr Val Ile Thr Leu Ala Asp Trp Tyr His  
 165 170 175  
 Thr Ala Ala Lys Leu Gly Pro Ala Phe Pro Ala Gly Pro Asp Ser Val  
 180 185 190  
 Leu Ile Asn Gly Leu Gly Arg Phe Ser Gly Asp Gly Gly Ala Thr  
 195 200 205  
 Asn Leu Thr Val Ile Thr Val Thr Gln Gly Lys Arg Tyr Arg Phe Arg  
 210 215 220  
 Leu Val Ser Ile Ser Cys Asp Pro Asn Phe Thr Phe Ser Ile Asp Gly  
 225 230 235 240  
 His Asn Met Thr Ile Ile Glu Val Asp Gly Val Asn His Glu Ala Leu  
 245 250 255  
 Asp Val Asp Ser Ile Gln Ile Phe Ala Gly Gln Arg Tyr Ser Phe Ile  
 260 265 270  
 Leu Asn Ala Asn Gln Ser Ile Asp Asn Tyr Trp Ile Arg Ala Ile Pro  
 275 280 285  
 Asn Thr Gly Thr Thr Asp Thr Thr Gly Gly Val Asn Ser Ala Ile Leu  
 290 295 300  
 Arg Tyr Asp Thr Ala Glu Asp Ile Glu Pro Thr Thr Asn Ala Thr Thr  
 305 310 315 320  
 Ser Val Ile Pro Leu Thr Glu Thr Asp Leu Val Pro Leu Asp Asn Pro  
 325 330 335  
 Ala Ala Pro Gly Asp Pro Gln Val Gly Gly Val Asp Leu Ala Met Ser  
 340 345 350  
 Leu Asp Phe Ser Phe Asn Gly Ser Asn Phe Phe Ile Asn Asn Glu Thr  
 355 360 365  
 Phe Val Pro Pro Thr Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala  
 370 375 380  
 Gln Asp Ala Ala Ser Leu Leu Pro Asn Gly Ser Val Tyr Thr Leu Pro  
 385 390 395 400  
 Ser Asn Ser Thr Ile Glu Ile Ser Phe Pro Ile Ile Thr Thr Asp Gly  
 405 410 415  
 Val Leu Asn Ala Pro Gly Ala Pro His Pro Phe His Leu His Gly His  
 420 425 430  
 Thr Phe Ser Val Val Arg Ser Ala Gly Ser Ser Thr Phe Asn Tyr Ala  
 435 440 445  
 Asn Pro Val Arg Arg Asp Thr Val Ser Thr Gly Asn Ser Gly Asp Asn  
 450 455 460  
 Val Thr Ile Arg Phe Thr Thr Asp Asn Pro Gly Pro Trp Phe Leu His  
 465 470 475 480  
 Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val Trp Gly  
 485 490 495  
 Glu Asp Thr Ala Asp Thr Ala Ser Ala Asn Pro Val Pro Thr Ala Trp  
 500 505 510  
 Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Ser Ser Asp Leu  
 515 520 525



Applicant's or agent's file reference number	4185.204-WO	International application to be assigned	PCT/US 95/07536
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13 bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>55</u> line <u>4</u>	
B. IDENTIFICATION OF <span style="float: right;">Further deposits are identified on an additional sheet <input checked="" type="checkbox"/></span>	
Name of depository institution Agricultural Research Service Patent Culture Collection (NRRL)	
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Date of deposit May 25, 1995	Accession Number NRRL B-21263
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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A. The indications made below relate to the microorganism referred to in the description on page <u>55</u> , line <u>6</u>	
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A. The indications made below relate to the microorganism referred to in the description on page <u>55</u> , line <u>11</u>	
B. IDENTIFICATION OF <span style="float: right;">Further deposits are identified on an additional sheet <input checked="" type="checkbox"/></span>	
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A. The indications made below relate to the microorganism referred to in the description on page <u>55</u> , line <u>14</u>	
B. IDENTIFICATION OF <span style="float: right;">Further deposits are identified on an additional sheet <input checked="" type="checkbox"/></span>	
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Address of depository institution (including postal code and country)  Northern Regional Research Center 1815 University Street Peoria, IL 61604, US	
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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A. The indications made below relate to the microorganism referred to in the description on page 55, line 16	
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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A. The indications made below relate to the microorganism referred to in the description on page <u>55</u> , line <u>18</u>	
B. IDENTIFICATION OF <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
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Address of depository institution (including postal code and country)  Northern Regional Research Center 1815 University Street Peoria, IL 61604, US	
Date of deposit May 25, 1995	Accession Number NRRL B-21267
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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What we claim is:

1. A DNA construct containing a sequence encoding a *Polyporus* laccase.
- 5 2. The construct of Claim 1 which comprises a sequence encoding a *Polyporus pinsitus* laccase.
3. The construct of Claim 1 which comprises a nucleic acid  
10 sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.
4. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 1.
- 15 5. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 4.
- 20 6. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 3.
7. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID  
25 NO. 6.
8. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 5.
- 30 9. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 8.

10. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 7.
11. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 10.
12. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 9.
13. The construct of Claim 1, which comprises the nucleic acid sequence selected from those contained in NRRL B-21263, 21264, 21265, 21266, 21267, and 21268.
14. A substantially pure *Polyporus* laccase enzyme.
15. The enzyme of Claim 14 which is a *Polyporus pinsitus* laccase.
16. The enzyme of Claim 14 which comprises the amino acid sequence selected from the group consisting of the sequences depicted in SEQ ID NOS. 4, 6, 8, and 10 or a sequence with at least about 80% homology thereto.
17. A recombinant vector comprising an DNA construct containing a sequence encoding a *Polyporus* laccase.
18. The vector of Claim 17 in which the construct is operably linked to a promoter sequence.
19. The vector of Claim 18 in which the promoter is a fungal or yeast promoter.



20. The vector of Claim 19 in which the promoter is the TAKA amylase promoter of *Aspergillus oryzae*.

21. The vector of Claim 18 in which the promoter is the glucoamylase (*glaA*) promoter of *Aspergillus niger* or *Aspergillus awamori*.

22. The vector of Claim 17 which also comprises a selectable marker.

10

23. The vector of Claim 22 in which the selectable marker is selected from the group consisting of *amdS*, *pyrG*, *argB*, *niaD*, *sC*, *trpC* and *hygB*.

15 24. The vector of Claim 22 in which the selectable marker is the *amdS* marker of *Aspergillus nidulans* or *Aspergillus oryzae*, or the *pyrG* marker of *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus oryzae*.

20

25. The vector of Claim 18 which comprises both the TAKA amylase promoter of *Aspergillus oryzae* and the *amdS* or *pyrG* marker of *Aspergillus nidulans* or *Aspergillus oryzae*.

25 26. A recombinant host cell comprising a heterologous DNA construct containing a sequence encoding a *Polyporus* laccase.

27. The cell of Claim 26 which is a fungal cell.

30

28. The cell of Claim 27 which is an *Aspergillus* cell.

29. The cell of Claim 26 in which the construct is integrated into the host cell genome.

30. The cell of Claim 26 in which the construct is contained on a vector.
- 5 31. The cell of Claim 26 which comprises a construct containing a sequence encoding an amino acid sequence selected from the group consisting of those depicted in SEQ ID NOS. 2, 4, 6, 8, and 10.
- 10 32. A method for obtaining a laccase enzyme which comprises culturing a recombinant host cell comprising a DNA construct containing a nucleic acid sequence encoding a *Polyporus* laccase enzyme, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.
- 15 33. A method for obtaining a laccase enzyme which comprises culturing a recombinant *Aspergillus* host cell comprising a DNA construct containing a nucleic acid sequence encoding a *Polyporus*-like laccase enzyme, under conditions conducive to
- 20 expression of the enzyme, and recovering the enzyme from the culture.
34. A *Polyporus* enzyme obtained by the method of Claim 33.
- 25 35. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the substrate with a *Polyporus* laccase.
- 30 36. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a *Polyporus* laccase.

37. A method for oxidizing dyes or dye precursors which comprises contacting the dye or dye precursor with a *Polyporus* laccase.
- 5 38. A method for dyeing hair which comprises contacting a *Polyporus* laccase, in the presence or absence of at least one modifier, with at least one dye precursor, for a time and under conditions sufficient to permit oxidation of the dye precursor to a dye.
- 10 39. The method of claim 38 in which the dye precursor is selected from the group consisting of a diamine, aminophenol, and a phenol.
- 15 40. The method of claim 38, wherein the modifier, when used, is a meta-diamine, a meta-aminophenol or a polyphenol.
41. The method of claim 38 in which the dye precursor is a primary intermediate selected from the group consisting of  
20 an ortho- or para-diamine or aminophenol.
42. The method of claim 38 in which more than one dye precursor is used.
- 25 43. The method of claim 38 in which more than one modifier is used.
44. The method of claim 38 in which both a primary intermediate and a modifier are used.
- 30 45. A dye composition comprising a *Polyporus* laccase combined with at least one dye precursor.

46. A dye composition comprising a *Polyporus* laccase combined with at least one primary intermediate and at least one modifier.

5 47. A container containing a dye composition comprising a *Polyporus* laccase and at least one dye precursor in an oxygen-free atmosphere.

48. The container of claim 47 which contains at least one  
10 primary intermediate dye precursor combined with at least one modifier.

49. A method of polymerizing or oxidizing a phenolic or aniline compound which comprises contacting the phenolic or  
15 aniline compound with a *Polyporus* laccase.

FIG. 1A  
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563	573	583	592	601	610
ACGGGGCTTC ATTGTGCTAA TAATCGTCGT GTGCAG			CAC	TGG	CAC GGT TTC TTC CAG AAG
			His	Trp	His Gly Phe Phe Gln Lys
619	628	637	646	655	664
GGT	ACC AAC TGG GCC GAC GGT CCC GCC TTC ATC AAC CAG TGC CCG ATC TCA TCT				
Gly	Thr Asn Trp Ala Asp Gly Pro Ala Phe Ile Asn Gln Cys Pro Ile Ser Ser				
673	682	691	700	709	720
GGT	CAC TCG TTC CTG TAC GAC TTC CAG GTT CCT GAC CAG GCT G				GTAAGTACCG
Gly	His Ser Phe Leu Tyr Asp Phe Gln Val Pro Asp Gln Ala Gly				
730	740	750	760	770	779
TCGTTATGGA GTATACTGCC CATTGCTAAA CCACATGGTG AACAG GT				ACC TTC TGG TAT	
				Thr Phe Trp Tyr	
788	797	806	815	824	833
CAC	AGT CAC TTG TCT ACG CAG TAC TGT GAT GGT TTG AGG GGT CCG TTC GTT GTT				
His	Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Phe Val Val				
842	851	860	869	878	889
TAC	GAC CCG AAT GAC CCG GCC GCC GAC CTG TAC GAC GTC GAC AAC G				GTAAGGACGA
Tyr	Asp Pro Asn Asp Pro Ala Ala Asp Leu Tyr Asp Val Asp Asn Asp				
899	909	919	929	940	949
ATTCCAACCG TAAATACTTG CTTACTGATA CTCTCGATG AATTAG AC				GAC ACT GTC ATT	
				Asp Thr Val Ile	
958	967	976	985	994	1009
ACC	CTT GTG GAT TGG TAC CAC GTC GCC GCG AAG CTG GCG GGG GCA TTC CC				GTAAGTCCAT
Thr	Leu Val Asp Trp Tyr His Val Ala Ala Lys Leu Gly Pro Ala Phe Pro				

FIG.1B

1019	1029	1039	1049	1060	1069
GAGTATTCTG CTGTTGAATC TGTCTTA <u>ACT</u> GTGCATATCA G T				CTC	GCC ACC
				Leu Gly	Ala Asp Ala Thr
1078	1087	1096	1105	1114	1123
CTC	ATC	AAC	GGT	AAG	GGA
Leu	Ile	Asn	Gly	Lys	Gly
CGC	TCC	CCC	AGC	ACG	ACC
Arg	Ser	Pro	Ser	Thr	Thr
ACC	ACC	GCG	GAC	CTC	TCA
Thr	Thr	Ala	Asp	Leu	Ser
GTT					Val
1132	1141	1156	1166	1176	1186
ATC	AGC	GTC	ACC	CCG	GGT
Ile	Ser	Val	Thr	Pro	Gly
AAA	CG	GTATGCTATA	TCTTATCTTA	TCTGATGGCA	TTTCTCTGAG
Lys	Arg				
1196	1207	1216	1225	1234	
ACATTCTCCA	G C	TAC	CGT	TTC	CGC
		Tyr	Arg	Phe	Arg
GTG	TCC	CTG	TCG	TGC	GAC
Val	Ser	Leu	Ser	Cys	Asp
ACC	AAC	TAC			
Pro	Asn	Tyr			
1243	1252	1261	1270	1279	1288
ACG	TTC	AGC	ATC	GAT	GGT
Thr	Phe	Ser	Ile	Asp	Gly
CAC	AAC	ATG	ACG	ATC	ATC
His	Asn	MET	Thr	Ile	Ile
GAG	ACC	GAC	TCA	ATC	AAC
Glu	Thr	Asp	Ser	Ile	Asn
1297	1306	1315	1324	1333	1342
ACC	GCG	CCC	CTC	GTC	GTC
Thr	Ala	Pro	Leu	Val	Val
GAC	TCC	ATT	CAG	ATC	TTC
Asp	Ser	Ile	Gln	Ile	Phe
CCC	CAG	CGT	TAC	TGC	
Ala	Gln	Arg	Tyr	Ser	
1351	1364	1374	1384	1394	1404
TTC	GTG	GTAAGTTCCA	TTCATCCTCT	AACGTTGGTC	GCTGTTAGTG
Phe	Val				
1414	1423	1432	1441	1450	1459
CTC	GAG	GCC	AAC	CAG	GCC
Leu	Glu	Ala	Asn	Gln	Ala
GTC	GAC	AAC	TAC	TGG	ATT
Val	Asp	Asn	Tyr	Trp	Ile
CGC	GCC	AAC	CCG	AAC	TTC
Arg	Ala	Asn	Pro	Asn	Phe

FIG.1C

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1468	1477	1486	1495	1504	1513
GGT AAC GTC GGG TTC ACC GGC GGC ATT AAC TCG GCT ATC CTC CGC TAC GAT GGT					
Gly Asn Val Gly Phe Thr Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr Asp Gly					
1522	1531	1540	1549	1558	1567
GCC GCT GCC GTG GAG CCC ACC ACA ACG CAA ACC ACG TCG ACT GCG CCG CTC AAC					
Ala Ala Ala Val Glu Pro Thr Thr Thr Gln Thr Thr Ser Thr Ala Pro Leu Asn					
1576	1585	1594	1603	1619	1629
GAG GTC AAC CTG CAC CCG CTG GTT ACC ACC GCT GTG GTATGTAATA TTGTCGGTAA					
Glu Val Asn Leu His Pro Leu Val Thr Thr Ala Val					
1639	1649	1659	1669	1678	1687
TGTAATACAT TGTTCGCTGAC CTOGACCCCC ACAG CCT GGC TCG CCC GTC GCT GGT GGT					
			Pro Gly Ser Pro Val Ala Gly Gly		
1696	1705	1714	1723	1732	1741
GTC GAC CTG GCC ATC AAC ATG GCG TTC AAC TTC AAC GGC ACC AAC TTC TTC ATC					
Val Asp Leu Ala Ile Asn MET Ala Phe Asn Phe Asn Gly Thr Asn Phe Phe Ile					
1750	1759	1768	1777	1786	1795
AAC GGC ACG TCT TTC ACG CCC CCG ACC GTG CCT GTC CTG CTC CAG ATC ATC AGC					
Asn Gly Thr Ser Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln Ile Ile Ser					
1804	1813	1822	1831	1840	1849
GGC GCG CAG AAC GCG CAG GAC CTC CTG CCC TCC GGT AGC GTC TAC TCG CTT CCC					
Gly Ala Gln Asn Ala Gln Asp Leu Leu Pro Ser Gly Ser Val Tyr Ser Leu Pro					
1858	1867	1876	1885	1894	1903
TCG AAC GCC GAC ATC GAG ATC TCC TTC CCC GCC ACC GCC GCC GCC CCC GGT GCG					
Ser Asn Ala Asp Ile Glu Ile Ser Phe Pro Ala Thr Ala Ala Ala Pro Gly Ala					

FIG. 1D

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1912			1921			1930			1939			1948			1957			
CCC	CAC	CCC	TTC	CAC	TTG	CAC	GGG	CAC	GCG	TTC	GCG	GTC	GTC	CGC	AGC	GCC	GGC	
Pro	His	Pro	Phe	His	Leu	His	Gly	His	Ala	Phe	Ala	Val	Val	Arg	Ser	Ala	Gly	
1966			1975			1984			1993			2002			2011			
AGC	ACG	GT	TAC	AAC	TAC	GAC	AAC	CCC	ATC	TTC	CGC	GAC	TC	GTC	AGC	ACG	GGG	
Ser	Thr	Val	Tyr	Asn	Tyr	Asp	Asn	Pro	Ile	Phe	Arg	Asp	Val	Val	Ser	Thr	Gly	
2020			2029			2038			2047			2056			2065			
ACG	CCT	GCG	GCC	GGT	GAC	AAC	GTC	ACC	ATC	CGC	TTC	CGC	ACC	GAC	AAC	CCC	GGC	
Thr	Pro	Ala	Ala	Gly	Asp	Asn	Val	Thr	Ile	Arg	Phe	Arg	Thr	Asp	Asn	Pro	Gly	
2074			2083			2092			2101			2110			2119			
CCG	TGG	TTC	CTC	CAC	TGC	CAC	ATC	GAC	TTC	CAC	CTC	GAG	GCC	GGC	TTC	GCC	GTC	
Pro	Trp	Phe	Leu	His	Cys	His	Ile	Asp	Phe	His	Leu	Glu	Ala	Gly	Phe	Ala	Val	
2128			2137			2146			2155			2164			2173			
GTG	TTC	GCG	GAG	GAC	ATC	CCC	GAC	GTC	GCG	TCG	GCG	AAC	CCC	GTC	CCC	CAG	GCG	
Val	Phe	Ala	Glu	Asp	Ile	Pro	Asp	Val	Ala	Ser	Ala	Asn	Pro	Val	Pro	Gln	Ala	
2182			2191			2200			2209			2218			2231			
TGG	TCC	GAC	CTC	TGT	CCG	ACC	TAC	GAC	GCG	CTC	GAC	CCG	AGC	GAC	CAG	TAAATGGCTT		
Trp	Ser	Asp	Leu	Cys	Pro	Thr	Tyr	Asp	Ala	Leu	Asp	Pro	Ser	Asp	Gln			
2241			2251			2261			2271			2281			2291			2301
GCGCCGGTCG ATGATAGGAT ATGGACGGTG AGTTCGCACT TGCAATACGG ACTCTCGCCT CATTATGGTT																		
2311			2321			2331			2341			2351			2361			2371
ACACACTCGC TCTGGATCTC TCGCCTGTG ACAGAACAAA CTTGTATAAT TCGCTTAATG GTTGAAACAA																		
2381			2391			2401			2411									
ATGGAATATT GGGGTACTAT GCACGCATCT CGCTGGGTGA GCTTTCGT																		

FIG.1E  
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10	20	30	40	50	60	70
GCGGCGCACA AACCGTGGGA GCCAACACAC TCCCGTCCAC TCTCACA CTG GCCAGATTCC CGCGACCGCC						
80	90	100	110	120	130	140
GCCTTTCAGG CCCAAACAGA TCTGGCAGGT TTCGATGGCG CACGCCGCCG TGCCTGCCGG ATTCAATTGT						
150	160	170	180	190	200	210
GCGCCAGTCC GGCATCCGGA TGGCTCTACC AGCGCGGTTG ACTGGAAGAG AACACCGAGG TCATGCATTC						
220	230	240	250	260	270	280
TGGCCAAGTG CGGCCAAAGG ACGCTCCCT GGTGCGGATA CTTAAAGGCC GGCGCGGGA GGCCTGTCTA						
290	300	310	320	330	340	350
CCAAGCTCAA GCTCGCCTTG GGTCCCACT CTCGCCACC CTCCTCTCC CCCACACAGT CGCTCCATAG						
360	369	378	387	396	405	
<div style="display: flex; align-items: center;"> <div style="margin-right: 10px;">CACCGTCGGC</div> <div style="display: flex; align-items: center;"> <div style="margin-right: 5px;">GCC</div> <div style="display: flex; align-items: center;"> <div style="margin-right: 5px;">ATG</div> <div style="margin-right: 5px;">GGT</div> <div style="margin-right: 5px;">CTG</div> <div style="margin-right: 5px;">CAG</div> <div style="margin-right: 5px;">CGA</div> <div style="margin-right: 5px;">TTC</div> <div style="margin-right: 5px;">AGC</div> <div style="margin-right: 5px;">TTC</div> <div style="margin-right: 5px;">TTC</div> <div style="margin-right: 5px;">GTC</div> <div style="margin-right: 5px;">ACC</div> <div style="margin-right: 5px;">CTC</div> <div style="margin-right: 5px;">GCG</div> <div style="margin-right: 5px;">CTC</div> </div> </div> </div>						
MET Gly Leu Gln Arg Phe Ser Phe Phe Val Thr Leu Ala Leu						
414	423	432	441	450	459	
<div style="display: flex; align-items: center;"> <div style="margin-right: 10px;">GTC GCT CGC TCT CTT GCA GCC ATC GGG CCG GTG GCG AGC CTC GTC GTC GCG AAC</div> </div>						
Val Ala Arg Ser Leu Ala Ala Ile Gly Pro Val Ala Ser Leu Val Val Ala Asn						
468	477	486	495	504	513	
<div style="display: flex; align-items: center;"> <div style="margin-right: 10px;">GCC CCC GTC TCG CCC GAC GGC TTC CTT CCG GAT GCC ATC GTG GTC AAC GGC GTG</div> </div>						
Ala Pro Val Ser Pro Asp Gly Phe Leu Arg Asp Ala Ile Val Val Asn Gly Val						
522	531	540	553	563	573	
<div style="display: flex; align-items: center;"> <div style="margin-right: 10px;">GTC CCT TCC CCG CTC ATC ACC GGG AAG AAG GTCGGCGTGT TCGTCGTCGT CCTACTCCT</div> </div>						
Val Pro Ser Pro Leu Ile Thr Gly Lys Lys						

FIG.2A

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583	592	601	610	619	628
TGCTGACAGC GATCTACAG GGA GAC CGC GTC CAG CTC AAC GTC GTC GAC ACC TTG					
Gly Asp Arg Phe Gln Leu Asn Val Val Asp Thr Leu					
637	646	655	671	681	691
ACC AAC CAC AGC ATG CTC AAG TCC ACT AGT ATC GTAAGTGTGA CGATCCGAAT GTGACATCAA					
Thr Asn His Ser MET Leu Lys Ser Thr Ser Ile					
701	711	721	730	739	748
TCGGGGCTAA TTAACCGCGC ACAG CAC TGG CAC GGC TTC TTC CAG GCA GGC ACC AAC					
His Trp His Gly Phe Phe Gln Ala Gly Thr Asn					
757	766	775	784	793	802
TGG GCA GAA GGA CCC GCG TTC GTC AAC CAG TGC CCT ATT GCT TCC GGG CAT TCA					
Trp Ala Glu Gly Pro Ala Phe Val Asn Gln Cys Pro Ile Ala Ser Gly His Ser					
811	820	829	846	856	
TTC CTG TAC GAC TTC CAT GTG CCC GAC CAG GCA G GTAAGCAGGA TTTTCTGGGG					
Phe Leu Tyr Asp Phe His Val Pro Asp Gln Ala Gly					
866	876	886	896	905	914
TCCCGGTGTG ATGCAATGTT CTCATGCTCC GACGTGATCG ACAG GG ACG TTC TGG TAC CAC					
Thr Phe Trp Tyr His					
923	932	941	950	959	968
AGT CAT CTG TCT ACG CAG TAC TGT GAC GGG CTG CGG GGG CCG TTC GTC GTG TAC					
Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Phe Val Val Tyr					
977	986	995	1004	1013	1024
GAC CCC AAG GAC CCG CAC GCC AGC CGT TAC GAT GTT GAC AAT G GTACGTGGCG					
Asp Pro Lys Asp Pro His Ala Ser Arg Tyr Asp Val Asp Asn Glu					

FIG.2B

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1034	1044	1054	1064	1075	1084
CACGGAGTAT ATCACACAGC ATCGGTTGAC GTCGGGCCAA CAGAG				AGC	ACG GTC ATC ACG
				Ser	Thr Val Ile Thr
1093	1102	1111	1120	1129	1141
TTG	ACC GAC	TGG TAC CAC	ACC GCT GCC CGG	CTC GGT CCC AAG	TTC CC GTAAGCTCGC
Leu	Thr Asp	Trp Tyr His	Thr Ala Ala Arg	Leu Gly Pro Lys	Phe Pro
1151	1161	1171	1181	1190	1199
AATGGCTTAG TGTTACACAGG TTCTTTGCTT ATGTTGCTTC GATAG				A	CTC GGC GCG GAC GCC
				Leu	Gly Ala Asp Ala
1208	1217	1226	1235	1244	1253
ACG	CTC ATC	AAC GGT CTG	GGG CGG TCT	GCC TCC ACT	CCC ACC GCT
Thr	Leu Ile	Asp Gly Leu	Gly Arg Ser	Ala Ser Thr	Pro Thr Ala
1262	1271	1280	1292	1302	1312
GTG	ATC AAC	GTC CAG CAC	GGA AAG CG	GTGAGCATTC	TCTTGATGC
Val	Ile Asn	Val Gln His	Gly Lys Arg	CATTTC AATG	
1322	1332	1341	1351	1360	1369
CTTTGTGCTG ACCTATCGGA ACCGCGCAG			C	TAC CGC TTC CGT	CTC GTT TCG
			Tyr	Arg Phe Arg	Leu Val Ser Ile Ser
1378	1387	1396	1405	1414	1423
TGT	GAC CCG	AAC TAC ACG	TTC AGC ATC	GAC GGG CAC	AAC CTG
Cys	Asp Pro	Asn Tyr Thr	Phe Ser Ile	Asp Gly His	Asn Leu Thr
1432	1441	1450	1459	1468	1477
GTC	GAC GGC	ATC AAT AGC	CAG CCT CTC	CTT GTC GAC	TCT ATC
Val	Asp Gly	Ile Asn Ser	Gln Pro Leu	Leu Val Asp	Ser Ile Gln
1486	1495	1508	1518	1528	1538
GCA	CAG CGC	TAC TCC TTC	GTG GTAAGTCCTG	GCTTGTCGAT	GCTCCAAAGT
Ala	Gln Arg	Tyr Ser Phe	Val	GGCCTCACTC	

FIG. 2C  
89-58

1548	1559	1568	1577	1586		
ATATACTTTC GTTAG TTG AAT GCG AAT CAA ACG GTG GGC AAC TAC TGG GTT CGT						
Leu Asn Ala Asn Gln Thr Val Gly Asn Tyr Trp Val Arg						
1595	1604	1613	1622	1631	1640	
GCG AAC CCG AAC TTC GGA ACG GTT GGG TTC GCC GGG GGG ATC AAC TCC GCC ATC						
Ala Asn Pro Asn Phe Gly Thr Val Gly Phe Ala Gly Gly Ile Asn Ser Ala Ile						
1649	1658	1667	1676	1685	1694	
TTG CGC TAC CAG GGC GCA CCG GTC GCC GAG CCT ACC ACG ACC CAG ACG CCG TCG						
Leu Arg Tyr Gln Gly Ala Pro Val Ala Glu Pro Thr Thr Thr Gln Thr Pro Ser						
1703	1712	1721	1730	1739	1748	1761
GTG ATC CCG CTC ATC GAG ACG AAC TTG CAC CCG CTC GCG CGC ATG CCA GTG GTATGTCTCT						
Val Ile Pro Leu Ile Glu Thr Asn Leu His Pro Leu Ala Arg MET Pro Val						
1771	1781	1791	1801	1811	1821	
TTTTCTGATC ATCTGAGTTG CCCGTTGTTG ACCGCATTAT GTGTTACTAT CTAG CCT GGC ACG						
Pro Gly Ser						
1830	1839	1848	1857	1866	1882	
CCG ACA CCC GGG GGC GTC GAC AAG GCG CTC AAC CTC GCG TTT AAC TTC GTAAGTATCT						
Pro Thr Pro Gly Gly Val Asp Lys Ala Leu Asn Leu Ala Phe Asn Phe						
1892	1902	1912	1922	1931	1940	
CTACTACTT GGCTGGAGGC TGGTCGCTGA TCATACGGTG CTTCAG AAC GGC ACC AAC TTC						
Asn Gly Thr Asn Phe						
1949	1958	1967	1976	1985	1994	
TTC ATC AAC AAC GCG ACT TTC ACG CCG CCG ACC GTC CCG GTA CTC CTC CAG ATT						
Phe Ile Asn Asn Ala Thr Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln Ile						

FIG.2D

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SUBSTITUTE SHEET (RULE 26)

2003	2012	2021	2030	2039	2048
CTG AGC GGT GCG CAG ACC GCA CAA GAC CTG CTC CCC GCA GGC TCT GTC TAC CCG					
Leu Ser Gly Ala Gln Thr Ala Gln Asp Leu Leu Pro Ala Gly Ser Val Tyr Pro					
2057	2066	2075	2084	2093	2102
CTC CCG GCC CAC TCC ACC ATC GAG ATC ACG CTG CCC GCG ACC GCC TTG GCC CCG					
Leu Pro Ala His Ser Thr Ile Glu Ile Thr Leu Pro Ala Thr Ala Leu Ala Pro					
2111	2120	2129	2145	2155	2165
GGT GCA CCG CAC CCC TTC CAC CTG CAC GGT GTATGTTCCC CTGCCTTCCC TTCITATCCC					
Gly Ala Pro His Pro Phe His Leu His Gly					
2175	2185	2195	2204	2213	2222
CGAACCAGTG CTCACGTCCG TCCCATCTAG CAC GCC TTC GCG GTC GTT CCG AGC GCG					
His Ala Phe Ala Val Val Arg Ser Ala					
2231	2240	2249	2258	2267	2276
GGG AGC ACC ACG TAT AAC TAC AAC GAC CCG ATC TTC CCG GAC GTC GTG ACC ACG					
Gly Ser Thr Thr Tyr Asn Tyr Asn Asp Pro Ile Phe Arg Asp Val Val Ser Thr					
2285	2294	2303	2312	2321	2330
GGC ACG CCC GCC GCG GGC GAC AAC GTC ACG ATC CCG TTC CAG ACG GAC AAC CCC					
Gly Thr Pro Ala Ala Gly Asp Asn Val Thr Ile Arg Phe Gln Thr Asp Asn Pro					
2339	2348	2357	2366	2375	2384
GGG CCG TGG TTC CTC CAC TGG CAC ATC GAC TTC CAC CTC GAC GCA GGC TTC GCG					
Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Asp Ala Gly Phe Ala					
2393	2402	2411	2420	2429	2438
ATC GTG TTC GCA GAG GAC GTT GCG GAC GTG AAG GCG GCG AAC CCG GTT CCG AAG					
Ile Val Phe Ala Glu Asp Val Ala Asp Val Lys Ala Ala Asn Pro Val Pro Lys					

FIG.2E

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2447	2456	2465	2474	2483		2499											
CGG	TGG	TCG	GAC	CTG	TGC	CCG	ATC	TAC	GAG	GGG	CTG	AGC	GAG	GCT	AAC	CAG	TGAGCGGAGG
Ala	Trp	Ser	Asp	Leu	Cys	Pro	Ile	Tyr	Asp	Gly	Leu	Ser	Glu	Ala	Asn	Gln	
2509	2519	2529	2539	2549	2559	2569											
CGGTGGTGTG	GACCGTAAAG	CTCGCGCGTC	CACCTGGGGG	GTTGAAGGTG	TTCTGATTGA	AATGGTCTTT											
2579	2589	2599	2609	2619	2629	2639											
GGGTTTATTT	GTTGTTATTG	TAACTCGGTT	CTCTACGCAA	GGACCGAGGA	TTGTATAGGA	TGAAGTAACT											
2649	2659	2669	2679	2689													
TTCTTAATGT	ATTATGATAT	CAATTGACGG	AGGCATGGAC	TCCGAAGTGT													

FIG.2F

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10 20 30 40 50 60 70  
 TTTCCCGACT AAACCAATCT CAGNCCGCTT CCTCCTAGGG AACCGAGCGA TGTGGCGGCC CTCTCTATCC  
 80 90 100 110 120 130 140  
 AAGCTGTCCA TAAGAAGACG TTCAAATGCC GCAGCAAGCG AGGAAATAAG CATCTAACAG TGTTTTTCCC  
 150 160 170 180 190 200 210  
 ATAGTCGCAT TTGCGCGGCC TGTGGGACCG ACGCCCTAG AGCGCTTTGG GAAACGTCCG AAGTGGCGGG  
 220 230 240 250 260 270 280  
 TGTATTTCGT GTAGACGAGA CGGTATTTGT CTCATCATTC CCGTGCTTCA GGTGACACA GCCCAAAGGT  
 290 300 310 320 330 340 350  
 CTATGTACGG CCCTTCACAT TCCCTGACAC ATTGACGCAA CCCTGGGTGC GCCTCGGACA GTGCCTCGGT  
 360 370 380 390 400 410 420  
 TGTAGTATCG GGACGCCCTA GGATGCAAGA TTGGAAGTCA CCAAGGCCCG AAGGGTATAA AATACCGAGA  
 430 440 450 460 470 480  
 GGTCTACCA CTCTGCATC TCCAGTCGCA GAGTTCCTCT CCCTTGCCAG CCACAGCTCG AG  
 491 500 509 518 527 536  
 > ATG TCC TTC TCT AGC CTT CGC CGT GCC TTG GTC TTC CTG GGT GCT TGC AGC AGT  
 MET Ser Phe Ser Ser Leu Arg Arg Ala Leu Val Phe Leu Gly Ala Cys Ser Ser  
 545 554 563 572 581 590  
 GCG CTG GCC TCC ATC GGC CCA GTC ACT GAG CTC GAC ATC GTT AAC AAG GTC ATC  
 Ala Leu Ala Ser Ile Gly Pro Val Thr Glu Leu Asp Ile Val Asn Lys Val Ile  
 599 608 617 626 635 644  
 GCC CCG GAT GGC GTC GCT CGT GAT ACA GTC CTC GCC GGG GGC ACG TTC CCG GGC  
 Ala Pro Asp Gly Val Ala Arg Asp Thr Val Leu Ala Gly Gly Thr Phe Pro Gly  
 653 662 675 685 695 705  
 CCA CTC ATC ACA GGA AAG AAG GTATGCTAAG TAGTCCCGCC CCCATCATCC TGTGGCTGAC  
 Pro Leu Ile Thr Gly Lys Lys

FIG.3A

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715	726	735	744	753	
GTTCGACGCC	GCCAG	GGT GAC AAC TTC CGC ATC AAC GTC GTC GAC AAG TTG GTT			
		Gly Asp Asn Phe Arg Ile Asn Val Val Asp Lys Leu Val			
762	771	780	789	799	809 819
AAC CAG ACT ATG CTG ACA TCC ACC ACC ATT	GTATGTC	ACT	AGCTCTCGCT	ATCTCGAGAC	
Asn Gln Thr MET Leu Thr Ser Thr Thr Ile					
829	839	848	857	866	875
CCGCTGACCG	ACAACATTG	CCGTAG	CAC TGG CAC GGG ATG TTC CAG CAT ACG ACG		
			His Trp His Gly MET Phe Gln His Thr Thr		
884	893	902	911	920	929
AAC TGG GCG GAT GGT CCC GCC TTT GTG ACT CAA TGC CCT ATC ACC ACT GGT GAT					
Asn Trp Ala Asp Gly Pro Ala Phe Val Thr Gln Cys Pro Ile Thr Thr Gly Asp					
938	947	956	965	976	986
GAT TTC CTG TAC AAC TTC CGC GTG CCC GAC CAG ACA G					
Asp Phe Leu Tyr Asn Phe Arg Val Pro Asp Gln Thr Gly					
996	1006	1016	1026	1035	1044
GTA	CTCAAAG	ACATCTCTAA	GCATTGCTA	CCTAG	GA ACG TAC TGG TAC CAT AGC CAT
					Thr Tyr Trp Tyr His Ser His
1053	1062	1071	1080	1089	1098
CTG GCC TTG CAG TAC TGT GAT GGG CTT CGC GGC CCC CTG GTG ATT TAC GAT CCC					
Leu Ala Leu Gln Tyr Cys Asp Gly Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro					
1107	1116	1125	1134	1145	1155
CAT GAT CCG CAG GCA TAC CTG TAT GAC GTC GAT GAC G					
His Asp Pro Gln Ala Tyr Leu Tyr Asp Val Asp Asp Glu					

FIG.3B

1165	1175	1185	1198	1207	
AAAACGGTTA ACTTCTAATT CTGTAAATAT CTTCATAG			AG	AGC	ACC GTT ATC ACT CTG
				Ser Thr Val	Ile Thr Leu
1216	1225	1234	1243	1252	1267
GCA GAC TGG TAC CAT ACC CCG GCG CCT CTG CTG CCG CCT GCC GC	GTACGCCTCC				
Ala Asp Trp Tyr His Thr Pro Ala Pro Leu Leu Pro Pro Ala Ala					
1277	1287	1297	1307	1317	1328
ACACATCTGC ACAGCGTTCC GTATCTCATA CCCTTAAAGT TTATCGGACA G C					ACT TTG ATT
					Thr Leu Ile
1337	1346	1355	1364	1373	1382
AAT GGC CTG GGT CCG TGC CCT GGC AAC CCC ACC GCC GAC CTA GCC GTC ATC GAA					
Asp Gly Leu Gly Arg Trp Pro Gly Asn Pro Thr Ala Asp Leu Ala Val Ile Glu					
1391	1409	1419	1429	1439	1449
GTC CAG CAC GGA AAG CG GTATGTCATA GCTCGGTTAT CTATTCATAC TCGCGGCCCTC GAAGCTAAAA					
Val Gln His Gly Lys Arg					
1459	1470	1479	1488	1497	
CCTTGTTCCA G C TAC CCG TTC CGA CTG GTC AGC ACC TCA TGC GAC CCC AAC TAC					
Tyr Arg Phe Arg Leu Val Ser Thr Ser Cys Asp Pro Asn Tyr					
1506	1515	1524	1533	1542	1551
AAC TTC ACT ATC GAT GGC CAC ACC ATG ACA ATC ATC GAG GCG GAT GGG CAG AAC					
Asn Phe Thr Ile Asp Gly His Thr MET Thr Ile Ile Glu Ala Asp Gly Gln Asn					
1560	1569	1578	1587	1596	1605
ACC CAG CCA CAC CAA GTC GAC GGA CTT CAG ATC TTC GCG GCA CAG CCG TAC TCC					
Thr Gln Pro His Gln Val Asp Gly Leu Gln Ile Phe Ala Ala Gln Arg Tyr Ser					

FIG.3C

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1614	1627	1637	1647	1657	1667	
TTC GTT GTATGTTTC CGCATTTCGG GAAAAGGAAT TCGCTGACA GCTCGAGTGT GCGTAG						
Phe Val						
1676	1685	1694	1703	1712	1721	
CTT AAC GCT AAC CAA GCG GTC AAC AAC TAC TGG ATC CGT GCG AAC CCT AAC CGT						
Leu Asn Ala Asn Gln Ala Val Asn Asn Tyr Trp Ile Arg Ala Asn Pro Asn Arg						
1730	1739	1748	1757	1766	1775	
GCT AAC ACT ACG GCG TTC GCC AAC GCG ATC AAC TCC GCC ATC CTG GCG TAC AAG						
Ala Asn Thr Thr Gly Phe Ala Asn Gly Ile Asn Ser Ala Ile Leu Arg Tyr Lys						
1784	1793	1802	1811	1820	1829	
GGG GCG CCG ATT AAG GAG CCT ACG ACG AAC CAG ACT ACC ATC CCG AAC TTT TTG						
Gly Ala Pro Ile Lys Glu Pro Thr Thr Asn Gln Thr Thr Ile Arg Asn Phe Leu						
1838	1847	1856	1865	1874	1884	1894
TGG GAG ACG GAC TTG CAC CCG CTC ACT GAC CCA CGT GCA GTAAGTTCTA CACAGTCACC						
Trp Glu Thr Asp Leu His Pro Leu Thr Asp Pro Arg Ala						
1904	1914	1924	1933	1942	1951	
AACGGTGAGC TGTGTCTGA TTGCACTGTG TTATAG CCT GGC CTT CCT TTC AAG GGG GGC						
Pro Gly Leu Pro Phe Lys Gly Gly						
1960	1969	1978	1987	1997	2007	2017
GTT GAC CAC GCT TTG AAC CTC AAC CTC ACT TTC GTACGTAGCG CCTCAGATAT CGAGTAGTCT						
Val Asp His Ala Leu Asn Leu Asn Leu Thr Phe						
2027	2037	2046	2055	2064	2073	
ATCTCCTGAC CGATTGACAC AAT CCA TCG GAG TTC TTC ATC AAC GAT GCC CCT TTC						
Asn Gly Ser Glu Phe Phe Ile Asn Asp Ala Pro Phe						

FIG.3D

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2082	2091	2100	2109	2118	2127
GTC CCT CCG ACT GTC CCG GTG CTA CTG CAG ATC CTG AAC GGA ACC CTC GAC GCG					
Val Pro Pro Thr Val Pro Val leu Leu Gln Ile Leu Asn Gly Thr Leu Asp Ala					
2136	2145	2154	2163	2172	2181
AAC GAC CTC CTG CCG CCC GGC AGC GTC TAC AAC CTT CCT CCG GAC TCC ACC ATC					
Asn Asp Leu Leu Pro Pro Gly Ser Val Tyr Asn Leu Pro Pro Asp Ser Thr Ile					
2190	2199	2208	2217	2226	2235
GAG CTG TCC ATT CCC GGA GGT GTG ACC GGT GGC CCG CAC CCA TTC CAT TTG CAC					
Glu Leu Ser Ile Pro Gly Gly Val Thr Gly Gly Pro His Pro Phe His Leu His					
2248	2258	2268	2278	2288	2297
GGG GTAATAATCT CTCTTTATAC TTTGGTCTCC CGATGCTGAC TTTCACCTGCT CATCTTCAG					
Gly					
2306	2315	2324	2333	2342	2351
CAC GCT TTC TCC GTC GTG CGT AGC GCC GGC AGC ACC GAA TAC AAC TAC GCG AAC					
His Ala Phe Ser Val Val Arg Ser Ala Gly Ser Thr Glu Tyr Asn Tyr Ala Asn					
2360	2369	2378	2387	2396	2405
CCG GTG AAG CGC GAC ACG GTC AGC ATT GGT CTT GCG GGC GAC AAC GTC ACC GTG					
Pro Val Lys Arg Asp Thr Val Ser Ile Gly Leu Ala Gly Asp Asn Val Thr Val					
2414	2424	2434	2444	2454	2464
CGC TTC GTG GTATGTTTTA CAGCCTCTCT ATCTCCGTGG GCGTTCGGAA GTTGACTGGG CCGGTAG					
Arg Phe Val					
2474	2483	2492	2501	2510	2519
ACC GAC AAC CCC GGC CCG TGG TTC CTC CAC TGT CAC ATC GAC TTC CAT TTG CAA					
Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Gln					

FIG.3E

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2528	2537	2546	2555	2564	2573
GCA GGC CTC GCC ATC GTG TTC GCG GAG GAC GCG CAG GAC ACG AAG CTT GTG AAC					
Ala Gly Leu Ala Ile Val Phe Ala Glu Asp Ala Gln Asp Thr Lys Leu Val Asn					

2582		2599	2609	2619	2629	2639
CCC GTC CCT G	GTACGTCTTC	TGGATGCATG	CGCTCCGCAC	AGTGACTCAT	CTTTTGCAAC	
Pro Val Pro Glu						

	2649	2658	2667	2676	2685
AG AG GAC TGG AAC AAG CTG TGC CCC ACC TTC GAT AAG GCG ATG AAC ATC ACC					
Asp Trp Asn Lys Leu Cys Pro Thr Phe Asp Lys Ala MET Asn Ile Thr					

2694	2704	2714	2724	2734	2744	2754
→						
GTT TCACCGATGC	GTGGCGCTCA	TGGTCATTTT	CTTGAATCT	TTGCATAGGG	CTGCAGCACC	
Val						

2764	2774	2784	2794	2804	2814	2824
CTGGATACTC	TTTCCCTTAG	CAGGATATTA	TTTAATGACC	CCTGCGTTTA	GTGCTTAGTT	AGCTTTACTA

2834	2844	2854	2864	2874	2884	2894
CTGGTTGTAA	TGTACGCAGC	ATGCGTAATT	CGGATAATGC	TATCAATGTG	TATATTATGA	CACGCGTCAT

2904	2914	2924	2934	2944	2954	2964
CGCGATGCT	TGAGTTGCAA	GGTCGGTTTC	CGATGCTCGA	CATAAACGTT	TCACTTACAT	ACACATTGGG

2974	2984	2994	3004	3014	3024	3034
TCTAGAACTG	GATCTATCCA	TGTATACAAA	AACTCCTCAT	ACAGCTGACT	GGGGCGCTCT	AGAGCATGGG

3044	3054	3064	3074	3084	3094	3104
TCCGATTGAT	CAGATGTCCG	GAACACGAGC	CTCCTGAGCT	CGAGGACTCT	GAGAAGCGGC	GGTGGTTCT

FIG.3F

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10	20	30	40	50	60	70
GCGCGTTGGC CGATTCATTA ATGCAGCTGG CACGACAGGT TTCCCGACTG GAAAGCGGGC AGTGAGCGCA						
80	90	100	110	120	130	140
ACGCAATTAA TGTGAGTTAG CTCACTCATT AGGCACCCCA GGCTTTACAC TTTATGCTTC CGGCTCGTAT						
150	160	170	180	190	200	210
GTTGTGTGGA ATTGTGAGCG GATAACAATT TCACACAGGA AACACCTATG ACATGATTAC GAATTCGGAT						
220	230	240	250	260	270	280
CGGCTTGCCC TCATTCTCC ATGTTCCCC GACCGAGCGG GCGCGTCAAT GGCCCGTTTG CGAACACATA						
290	300	310	320	330	340	350
TGCAGGATAA ACAGTGGAA ATATCAATGT GGCGGCGACA CAACCTCGCC GGCCGACACT CGACGCTGTT						
360	370	380	390	400	410	420
GATCATGATC ATGTCTTGTG AGCATTCTAT ACGCAGCCTT GGAAATCTCA GGCGAATTTG TCTGAATTGC						
430	440	450	460	470	480	490
GCTGGGAGGC TGGCAGCGCA GATCGGTGTG TCGGTGCACT AGCCGACGCA GCACCTGGCG GAAGCCGACA						
500	510	520	530	540	550	560
TCTCGGGTAC CACTTGATCT CCGCCAGATC ACTGCGGTTC CGCCATCGGC CGCGGGGCCC ATTCTGTGTG						
570	580	590	600	610	620	630
TGGCCTGTAG CACTCTGCAT TCAGGCTCAA CGTATCCATG CTAGAGGACC GTCCAGCTGT TGGCGCACGA						
640	650	660	670	680	690	700
TTCCGCGAGA AAGCTGTACA GGCAGATATA AGGATGTCCG TCCGTCAGAG ACTCGTCACT CACAAGCCTC						

FIG.4A  
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710            720            730            740            750            760            770  
 TTTTCTCTT CGCCTTTCCA GCCTCTTCCA ACGCCTGCCA TCGTCCTCTT AGTTGCTCG TCATTCTTT  
  
           780            790            799            808            817            826  
 CTGCGTAGTT AATC > GGC AGG TTC TCA TCT CTC TGC GCG CTC ACC GCC GTC ATC  
                  MET Gly Arg Phe Ser Ser Leu Cys Ala Leu Thr Ala Val Ile  
  
           835            844            853            862            871            880  
 CAC TCT TTT GGT CGT GTC TCC GCC GCT ATC GGG CCT GTG ACC GAC CTC ACC ATC  
 His Ser Phe Gly Arg Val Ser Ala Ala Ile Gly Pro Val Thr Asp Leu Thr Ile  
  
           889            898            907            916            925            934  
 TCC AAT GGG GAC GTT TCT CCC GAC GGC TTC ACT CGT GCC GCA GTG CTT GCA AAC  
 Ser Asn Gly Asp Val Ser Pro Asp Gly Phe Thr Arg Ala Ala Val Leu Ala Asn  
  
           943            952            961            970            980            990  
 GGC GTC TTC CCG GGT CCT CTT ATC ACG GGA AAC AAG GTACGTGGCA TCGTTTCAGT  
 Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys  
  
           1000            1010            1020            1029            1038            1047  
 CTACACCCTA CAAGCCTTCT AACTCTTTTA CCACAG GGC GAC AAC TTC CAG ATC AAT GTT  
                  Gly Asp Asn Phe Gln Ile Asn Val  
  
           1056            1065            1074            1083            1092            1105  
 ATC GAC AAC CTC TCT AAC GAG ACG ATG TTG AAG TCG ACC TCC ATC GTATGTGCTT  
 Ile Asp Asn Leu Ser Asn Glu Thr MET Leu Lys Ser Thr Ser Ile  
  
           1115            1125            1135            1145            1156            1165  
 CTA CTGCTTC TTAGTCTTGG CAATGGCTCA AGGTCTCCTC CGCAG CAT TGG CAC GGC TTC  
                  His Trp His Gly Phe

FIG.4B

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1174	1183	1192	1201	1210	1219
TTC CAG AAG GGT ACT AAC TGG GCT GAT GGA GCT GCC TTC GTC AAC CAG TGC CCT					
Phe Gln Lys gly thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro					
1228	1237	1246	1235	1264	
ATC GCG ACG GGG AAC TCT TTC CTT TAC GAC TTC ACC GCG ACG GAC CAA GCA G					
Ile Ala Thr Gly Asn Ser Phe Leu Tyr Asp Phe Thr Ala Thr Asp Gln Ala Gly					
1281	1291	1301	1311	1321	1331
GTCAGTGCCT GTGGCGCTTA TGTTTTCCCG TAATCAGCAG CTAACACTCC GCACCCACAG GC					
1342	1351	1360	1369	1378	1387
ACC TTC TGG TAC CAC AGT CAC TTG TCT ACG CAG TAC TGC GAT GGT TTG CCG GGC					
Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly					
1396	1405	1414	1423	1432	1441
CCG ATG GTC GTA TAC GAC CCG AGT GAC CCG CAT GCG GAC CTT TAC GAC GTC GAC					
Pro MET Val Val Tyr Asp Pro Ser Asp Pro His Ala Asp Leu Tyr Asp Val Asp					
1450	1459	1468	1477	1486	1495
GAC GAG ACC ACG ATC ATC ACG CTC TCT GAT TGG TAT CAC ACC GCT GCT TCG CTC					
Asp Glu Thr Thr Ile Ile Thr Leu Ser Asp Trp Tyr His Thr Ala Ala Ser Leu					
1504	1519	1529	1539	1549	1559
GGT GCT GCC TTC CC GTAAGTTTAC CCCAGCGCAC GGAGTTAAGA CCGATCTAA CTGTAATACG					
Gly Ala Ala Phe Pro					
1568	1577	1586	1604	1614	
TTCAG G ATT GGC TCG GAC TCT ACC CTG ATT AAC GG GTTGGCCGCT TCGCGGTGG					
Ile Gly Ser Asp Ser Thr Leu Ile Asn Gly					

FIG.4C

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1624	1633	1642	1651	1669
TGACAG C ACT GAC CTT GCG GTT ATC ACT GTC GAG CAG GGC AAG CG GTTAGTGATA				
Thr Asp Leu Ala Val Ile Thr Val Glu Gln Gly Lys Arg				
1679	1689	1699	1709	1719 1728
CCCTCTACAG TTGACACTGT GCCATTGCTG ACAGTACTCT CAG C TAC CGT ATG CGT CTT				
Tyr Arg MET Arg Leu				
1737	1746	1755	1764	1773 1782
CTC TCG CTG TCT TGC GAC CCC AAC TAT GTC TTC TCC ATT GAC GGC CAC AAC ATG				
Leu Ser Leu Ser Cys Asp Pro Asn Tyr Val Phe Ser Ile Asp Gly His Asn MET				
1791	1800	1809	1818	1827 1836
ACC ATC ATC GAG GCC GAC GCC GTC AAC CAC GAG CCC CTC ACG GTT GAC TCC ATC				
Thr Ile Ile Gln Ala Asp Ala Val Asn His Glu Pro Leu Thr Val Asp Ser Ile				
1845	1854	1863	1879	1889 1899
CAG ATC TAC GCC GGC CAA CGT TAC TCC TTC GTC GTACGTATTC CGAACAGCCA TGATCAGGCC				
Gln Ile Tyr Ala Gly Gln Arg Tyr Ser Phe Val				
1909	1919	1928	1937	1946 1955
AAGCCCGATG CTAACGCGCC TACCCTCAG CTT ACC GCT GAC CAG GAC ATC GAC AAC TAC				
Leu Thr Ala Asp Gln Asp Ile Asp Asn Tyr				
1964	1973	1982	1991	2000 2009
TTC ATC CGT GCC CTG CCC AGC GCC GGT ACC ACC TCG TTC GAC GGC GGC ATC AAC				
Phe Ile Arg Ala Leu Pro Ser Ala Gly Thr Thr Ser Phe Asp Gly Gly Ile Asn				
2018	2027	2036	2045	2054 2063
TCG GCT ATC CTG CGC TAC TCT GGT GCC TCC GAG GTT GAC CCG ACG ACC ACG GAG				
Ser Ala Ile Leu Arg Tyr Ser Gly Ala Ser Glu Val Asp Pro Thr Thr Thr Glu				

FIG.4D

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SUBSTITUTE SHEET (RULE 26)

2072	2081	2090	2099	2108	2117	
ACC	ACG	AGC	GTC	CTC	CCC	
Thr	Thr	Ser	Val	Leu	Pro	
2126	2136	2146	2156	2166	2176	
GCT	GCT	GTACGTGTA	TTCTGCGCTT	GCAAGGATCG	CACATACTAA	
Ala	Ala				Pro	
2185	2194	2203	2212	2221	2230	
GGT	GAC	CCC	AAC	ATT	GGC	
Gly	Asp	Pro	Asn	Ile	Gly	
2239	2248	2257	2266	2275	2284	
GAT	GGC	ACC	AAC	TTC	TTC	
Asp	Gly	Thr	Asn	Phe	Phe	
2293	2302	2311	2320	2329	2338	
GTC	CTC	CTC	CAG	ATT	CTT	
Val	Leu	Leu	Gln	Ile	Leu	
2347	2356	2365	2374	2383	2392	
GGT	AGT	CTC	TTC	GCG	GTC	
Gly	Ser	Leu	Phe	Ala	Val	
2401	2410	2419	2428	2437	2446	2456
ACC	GCG	ACG	AAC	GCT	CCC	
Thr	Ala	Thr	Asn	Ala	Pro	
2466	2476	2486	2496	2506	2515	
CATCTCATAT	GCTACGGAGC	TCCACGCTGA	CCGCCCTATA	G	CAC	
					His	

FIG.4E

2524	2533	2542	2551	2560	2569	
CGT ACC GCC GGC AGC ACG GAT ACG AAC TTC GTC AAC CCC GTC CGC CGC GAC GTC						
Arg Thr Ala Gly Ser Thr Asp Thr Asn Phe Val Asn Pro Val Arg Arg Asp Val						
2578	2587	2596	2605	2614	2624	
GTG AAC ACC GGT ACC GTC GGC GAC AAC GTC ACC ATC CGC TTC ACG GTACGCAGCA						
Val Asn Thr Gly Thr Val Gly Asp Asn Val Thr Ile Arg Phe Thr						
2634	2644	2654	2664	2673	2682	
CTCTCCTAAC ATTCCCACTG CGCGATCACT GACTCCTCGC CCACAG						
				ACT GAC AAC CCC GGC		
				Thr Asp Asn Pro Gly		
2691	2700	2709	2718	2727	2736	
CCC TGG TTC CTC CAC TGC CAC ATC GAC TTC CAC TTG GAG GCC GGT TTC GCC ATC						
Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile						
2745	2754	2763	2772	2781	2798	
GTC TTC AGC GAG GAC ACC GCC GAC GTC TCG AAC ACG ACC ACG CCC TCG A						
Val Phe Ser Glu Asp Thr Ala Asp Val Ser Asn Thr Thr Thr Pro Ser Thr						
2808	2818	2828	2838	2850	2859	
CTCCCGTGCC CATCTCCGCG CGCCTGACTA ACGAGCACCC CTTACAG						
				CT GCT TGG GAA GAT		
				Ala Trp Glu Asp		
2868	2877	2886	2895	2908	2918	
CTG TGC CCC ACG TAC AAC GCT CTT GAC TCA TCC GAC CTC						
Leu Cys Pro Thr Tyr Asn Ala Leu Asp Ser Ser Asp Leu						
2928	2938	2948	2958	2968	2978	2988
TCGCTACCTT AGTAGGTAGA CTTATGCACC GGACATTATC TACAATGGAC TTTAATTTGG GTTAACGGCC						
2998	3008	3018	3028	2038	3048	3058
GTTATACATA CGCGCAGTA GTATAAAGGT TCTCTGGATT GGTCCGACCT ACAGACTGCA ATTTTCGTGA						
3068	3078	3088	3098			
CCTATCAACT GTATATTGAA GCACGCAGT GAATGGAAAT AGAGACA						

FIG.4F

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SUBSTITUTE SHEET (RULE 26)

10	20	30	40	50	60	70	
CTCATAACTC TTCGTTCTA GCATGGGGC TCGGCACACC TGACAGACCC TTCGGGAGGC GAACTCGAAT							
80	90	100	110	120	130	140	
GCAGCGTACT CTATCNCACC TCCAGGAAAG GTAGGGATGG ACNCCGTGCA CCAACAAC TG TCTCTCCACC							
150	160	170	180	190	200	210	
AGCAACCATC CCTTGGATAT GTCTCCACAC ACCCGGTGTC TACAAGCGGG GATCTGTGCT GGTGAAGTGC							
220	230	240	250	260	270	280	
TGCTCCGGA GCGGCGCGG CGAGCGACCA GAACCGAAC CAGTGCTAGT GCCCGACACC CGCGAGACAA							
290	300	310	320	330	340	350	
<u>TTGTGCAGGG</u> TGAGTTATAT TCTTCGTGAG ACGGCGCTGC GCGTCGGCAC TGAAAGCGTC GCAGTTAGGT							
360	370	380	390	400	410	420	
GATGCAGCGG TCCGCGCTAT TTTTGACGTC TGGCAGCTAT CCTAAGCCGC GCCTCCATAC ACCCCAGGCG							
430	440	450	460	470	480	490	
CTCTCGTTTG CTATAGGTAT <u>AAATCCCTCA</u> GCTTCAGAGC GTCGATCCTC ATCCACACG ACACCCGTTT							
500	510	520	530	540	550		
CAGTCTTCTC GTAGCGCATT CCCTAGCCGC CCAGCCTCCG CTTTCGTTTT CAAC						<u>ATG</u> <u>GGC</u> <u>AAG</u> MET Gly Lys	
559	568	577	586	595	604		
<u>TAT</u>	<u>CAC</u>	<u>TCT</u>	<u>TTT</u>	<u>GTG</u>	<u>AAC</u>	<u>GTC</u>	<u>GTC</u>
Tyr	His	Ser	Phe	Val	Asn	Val	Val
<u>GCC</u>	<u>CTT</u>	<u>AGT</u>	<u>CTT</u>	<u>TCT</u>	<u>TTG</u>	<u>AGC</u>	<u>GGT</u>
Ala	Leu	Ser	Leu	Ser	Leu	Ser	Gly
<u>CGT</u>	<u>GTG</u>						
Arg	Val						
613	622	631	640	649	658		
<u>TTC</u>	<u>GGC</u>	<u>GCC</u>	<u>ATT</u>	<u>GGG</u>	<u>CCC</u>	<u>GTC</u>	<u>ACC</u>
Phe	Gly	Ala	Ile	Gly	Pro	Val	Thr
<u>GAC</u>	<u>TTG</u>	<u>ACT</u>	<u>ATC</u>	<u>TCT</u>	<u>AAC</u>	<u>GCC</u>	<u>GAT</u>
Asp	Leu	Thr	Ile	Ser	Asn	Ala	Asp
<u>GTT</u>	<u>ACG</u>						
Val	Thr						

FIG.5A

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667	676	685	694	703	712
CCT GAC GGC ATT ACT CGT GCT GCT GTC CTC GCG GGC GGC GTT TTC CCC GGC CCC					
Pro Asp Gly Ile Thr Arg Ala Ala Val Leu Ala Gly Gly Val Phe Pro Gly Pro					
721	730	743	753	763	773
CTC ATT ACC GGC AAC AAG GTGAGCCGCG AACCTTCTA CTAGCGCGCT CGTACGGTGC ACGTTACTG					
Leu Ile Thr Gly Asn Lys					
793	803	814	823	832	841
AAGCCACACT TTGCGCTGTC AACAG GGG GAT GAA TTC CAG ATC AAT GTC ATC GAC AAC					
		Gly Asp Glu Phe Gln Ile Asn Val Ile Asp Asn			
850	859	868	877	887	897
CTG ACC AAC GAG ACC ATG TTG AAG TCG ACC ACA ATC GTAAGGTGCT TGCTCCATA					
Leu Thr Asn Glu Thr MET Leu Lys Ser Thr Thr Ile					
907	917	927	938	947	956
ATTAAGCCCCG TCGCTGACTC GAAGTTTATC TGTAG CAC TGG CAT GGT ATC TTC CAG GCC					
			His Trp His Gly Ile Phe Gln Ala		
965	974	983	992	1001	1010
GGC ACC AAC TGG GCA GAC GGC GCG GCC TTC GTG AAC CAG TGC CCT ATC GCC ACG					
Gly Thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala Thr					
1019	1028	1037	1046		1063
GGA AAC TCG TTC TTG TAC GAC TTC ACC GTT CCT GAT CAA GCC G GTACGTTTAT					
Gly Asn Ser Phe Leu Tyr Asp Phe Thr Val Pro Asp Gln Ala Gly					
1073	1083	1093	1103	1112	1121
ACACTCCCT TTCTGCGCA TACTCTGACC CGCCGCTGGA TCAG GC ACC TTC TGG TAC CAC					
				Thr Phe Trp Tyr His	

FIG.5B

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SUBSTITUTE SHEET (RULE 26)

1130	1139	1148	1157	1166	1175
AGC CAC CTG TCC ACC CAG TAC TGT GAC GGC CTG CGC GGT CCT CTT GTG GTC TAC					
Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Leu Val Val Tyr					
1184	1193	1202	1211	1220	1231
GAC CCC GAC GAT CCC AAC GCG TCT CTT TAC GAC GTC GAT GAC G					GTAAGCAGGC
Asp Pro Asp Asp Pro Asn Ala Ser Leu Tyr Asp Val Asp Asp Asp					
1241	1251	1261	1271	1281	1290
TACTGTGGA CTTGTATGGA TGTATCTCAC GCTCCCCTAC AG AT ACT ACG GTT ATT ACG					
				Thr Thr Val Ile Thr	
1299	1308	1317	1326	1335	1347
CTT GCG GAC TGG TAC CAC ACT GCG GCG AAG CTG GCG CCT GCC TTC CC					GTGAGTCTAC
Leu Ala Asp Trp Tyr His Thr Ala Ala Lys Leu Gly Pro Ala Phe Pro					
1357	1367	1377	1387	1397	1408
TCTTCCTCGT GTGTTAACAT AGGTGACGGC CGCTGATACG AGAGCTACCA G C					GCG GGT CCG
					Ala Gly Pro
1417	1426	1435	1444	1453	1462
GAT AGC GTC TTG ATC AAT GGT CTT GGT CCG TTC TCC GGC GAT GGT GGA GGA GCG					
Asp Ser Val Leu Ile Asn Gly Leu Gly Arg Phe Ser Gly Asp Gly Gly Gly Ala					
1471	1480	1489	1498	1510	1520
ACA AAC CTC ACC GTG ATC ACC GTC ACG CAA GGC AAA CG					GTGAGTCCGC CCTGAGCTGG
Thr Asn Leu Thr Val Ile Thr Val Thr Gln Gly Lys Arg					
1530	1540	1550	1561	1570	1579
CCTCAATAGC GATATTGACG AGTCCATGCC CTCCCAG G					TAC CCG TTC CCG CTT GTG TCG
					Tyr Arg Phe Arg Leu Val Ser

FIG.5C

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1588	1597	1606	1615	1624	1633
ATC TCG TGC GAC CCC AAC TTC ACG TTC TCG ATC GAC GGG CAC AAC ATG ACC ATC					
Ile Ser Cys Asp Pro Asn Phe Thr Phe Ser Ile Asp Gly His Asn MET Thr Ile					
1642	1651	1660	1669	1678	1687
ATC GAG GTG GAC GGT GTC AAC CAC GAG GCC TTG GAC GTC GAC TCC ATT CAG ATT					
Ile Glu Val Asp Gly Val Asn His Glu Ala Leu Asp Val Asp Ser Ile Gln Ile					
1696	1705	1714	1724	1734	1744
TTT GCG GGG CAG CGG TAC TCC TTC ATC GTACGTTCCC TTGCCCTCGT GCTATATCCG					
Phe Ala Gly Gln Arg Tyr Ser Phe Ile					
1754	1764	1774	1785	1794	1803
CCCGTCTGCT CACAGAGGCT TCTATATCGC AG CTC AAC GCC AAC CAG TCC ATC GAC AAC					
Leu Asn Ala Asn Gln Ser Ile Asp Asn					
1812	1821	1830	1839	1848	1857
TAC TGG ATC CGC GCG ATC CCC AAC ACC GGT ACC ACC GAC ACC ACC GGC GGC GTG					
Tyr Trp Ile Arg Ala Ile Pro Asn Thr Gly Thr Thr Asp Thr Thr Gly Gly Val					
1866	1875	1884	1893	1902	1911
AAC TCT GCT ATT CTT CGC TAC GAC ACC GCA GAA GAT ATC GAG CCT ACG ACC AAC					
Asn Ser Ala Ile Leu Arg Tyr Asp Thr Ala Glu Asp Ile Glu Pro Thr Thr Asn					
1920	1929	1938	1947	1956	1965
GCG ACC ACC TCC GTC ATC CCT CTC ACC GAG ACG GAT CTG GTG CCG CTC GAC AAC					
Ala Thr Thr Ser Val Ile Pro Leu Thr Glu Thr Asp Leu Val Pro Leu Asp Asn					
1974	1983	1992	2001	2010	2019
CCT GCG GCT CCC GGT GAC CCC CAG GTC GGC GGT GTT GAC CTG GCT ATG AGT CTC					
Pro Ala Ala Pro Gly Asp Pro Gln Val Gly Gly Val Asp Leu Ala MET Ser Leu					

FIG.5D  
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2028	2041	2051	2061	2071	2081
GAC TTC TCC TTC	CTGAGTCCCA	CAGGACTCCG	CGCCATTTC	CTTATTTACG	CAGGAGTATT
Asp Phe Ser Phe					
2090	2099	2108	2117	2126	2135
GTTTCAG AAC GGT TCC AAC TTC TTT ATC AAC AAC GAG ACC TTC GTC CCG CCC ACA					
Asn Gly Ser Asn Phe Phe Ile Asn Asn Glu Thr Phe Val Pro Pro Thr					
2144	2153	2162	2171	2180	2189
GTT CCC GTG CTC CTG CAG ATT TTG AGT GGT GCG CAG GAC GCG GCG AGC CTG CTC					
Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala Gln Asp Ala Ala Ser Leu Leu					
2198	2207	2216	2225	2234	2243
CCC AAC GGG AGT GTC TAC ACA CTC CCT TCG AAC TCG ACC ATT GAG ATC TCG TTC					
Pro Asn Gly Ser Val Tyr Thr Leu Pro Ser Asn Ser Thr Ile Glu Ile Ser Phe					
2252	2261	2270	2279	2288	2297
CCC ATC ATC ACC ACC GAC GGT GTT CTG AAC GCG CCC GGT GCT CCG CAC CCG TTC					
Pro Ile Ile Thr Thr Asp Gly Val Leu Asn Ala Pro Gly Ala Pro His Pro Phe					
2306	2319	2329	2339	2349	2359
CAT CTC CAC GGC	GTAAGTCCTT	GCTTTCCTCA	GTGCCTCGCT	TCCACGACGT	CCACTGATCC
His Leu His Gly					
2369	2380	2389	2398	2407	2416
CACACATCCC ATGTGCAG	CAC ACC TTC TCG GTG GTG CCG AGC GCC GGG AGC TCG ACC				
His Thr Phe Ser Val Val Arg Ser Ala Gly Ser Ser Thr					
2425	2434	2443	2452	2461	2470
TTC AAC TAC GCC AAC CCA GTC CCG CCG GAC ACC GTC AGT ACT GGT AAC TCT GGC					
Phe Asn Tyr Ala Asn Pro Val Arg Arg Asp Thr Val Ser Thr Gly Asn Ser Gly					

FIG.5E  
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FIG. 5F

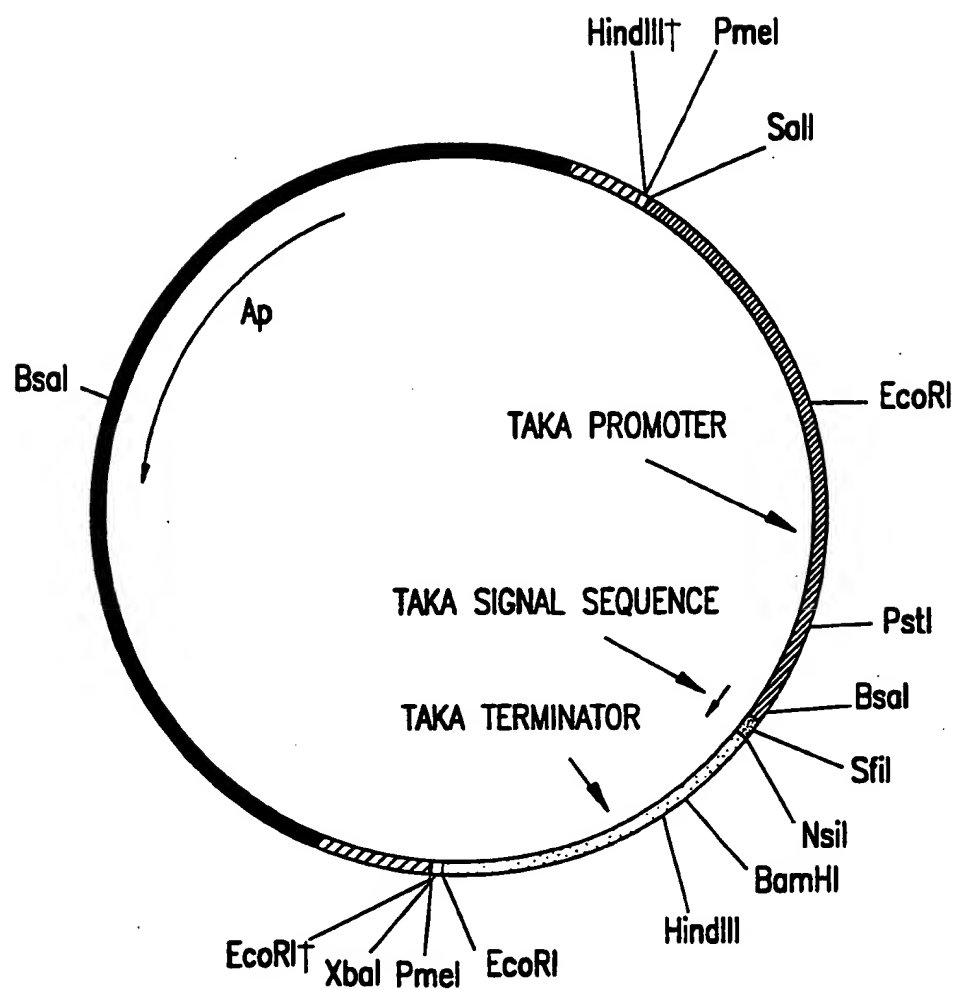


FIG.6

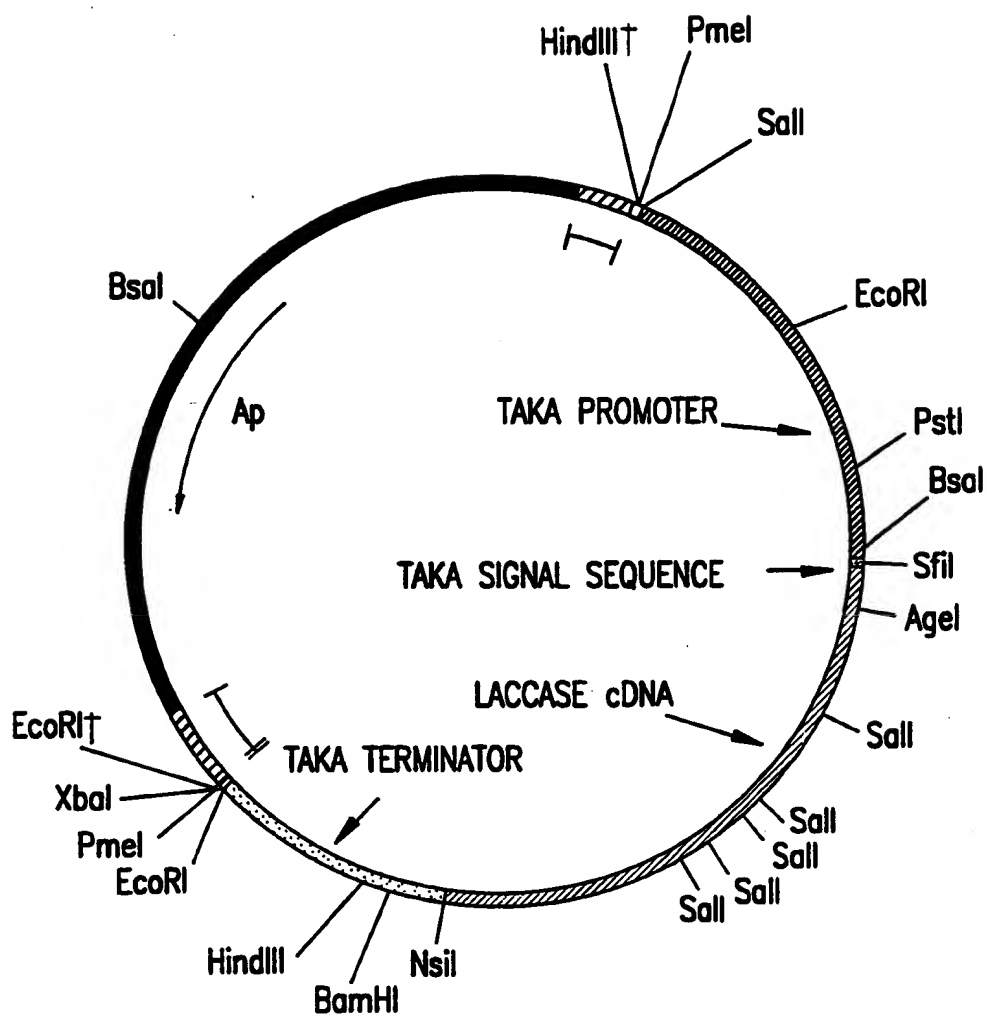


FIG.7

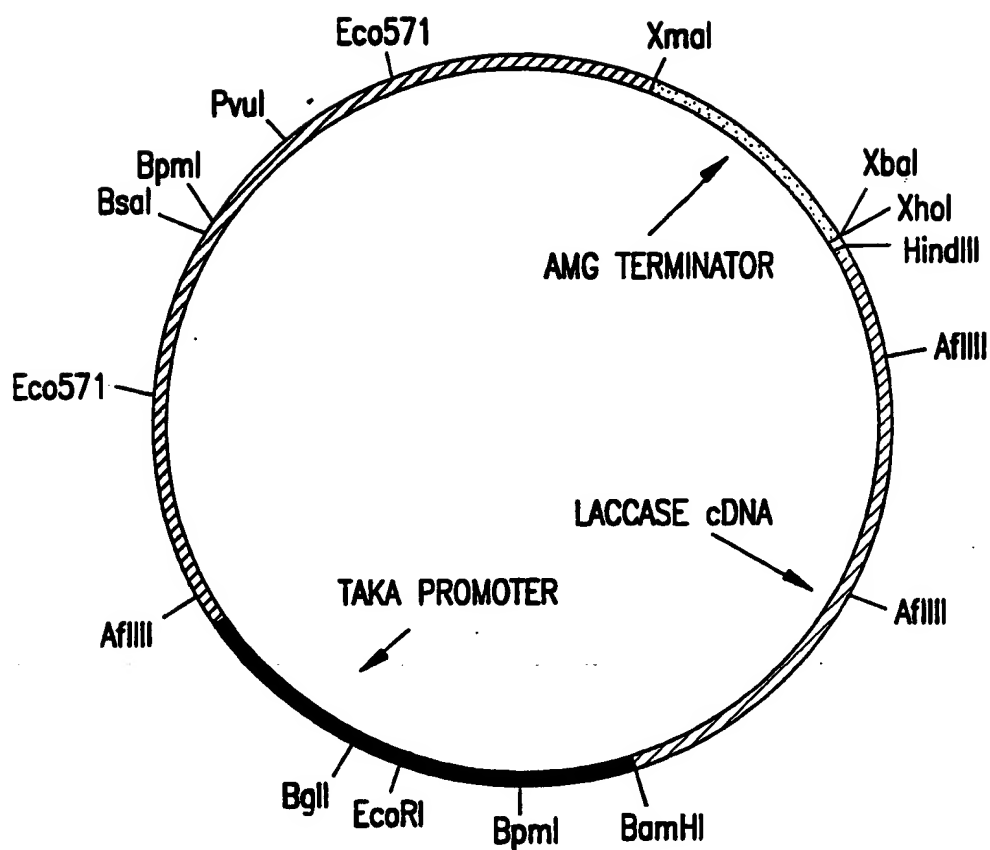


FIG.8

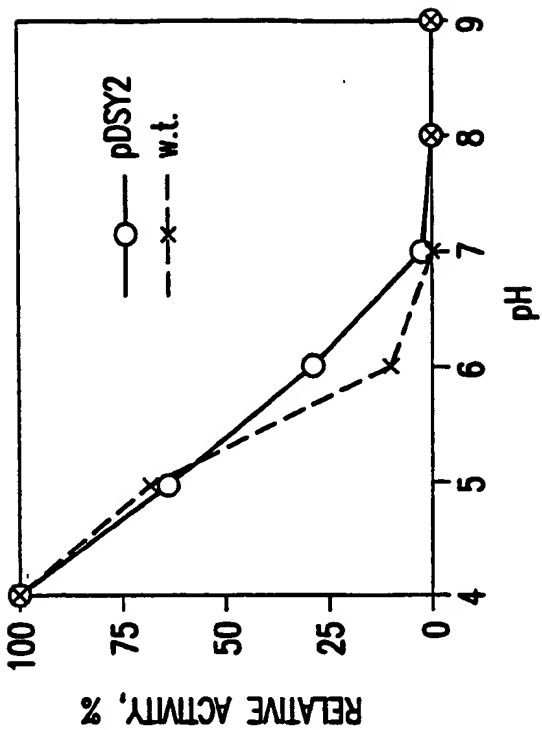


FIG. 9A

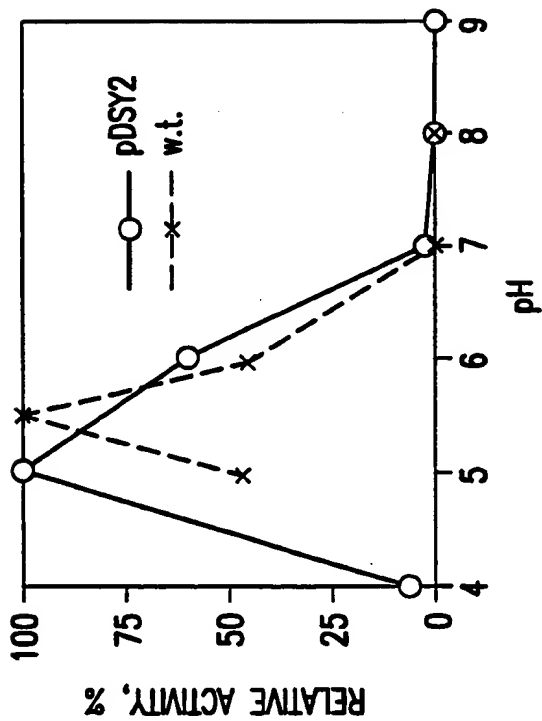


FIG. 9B

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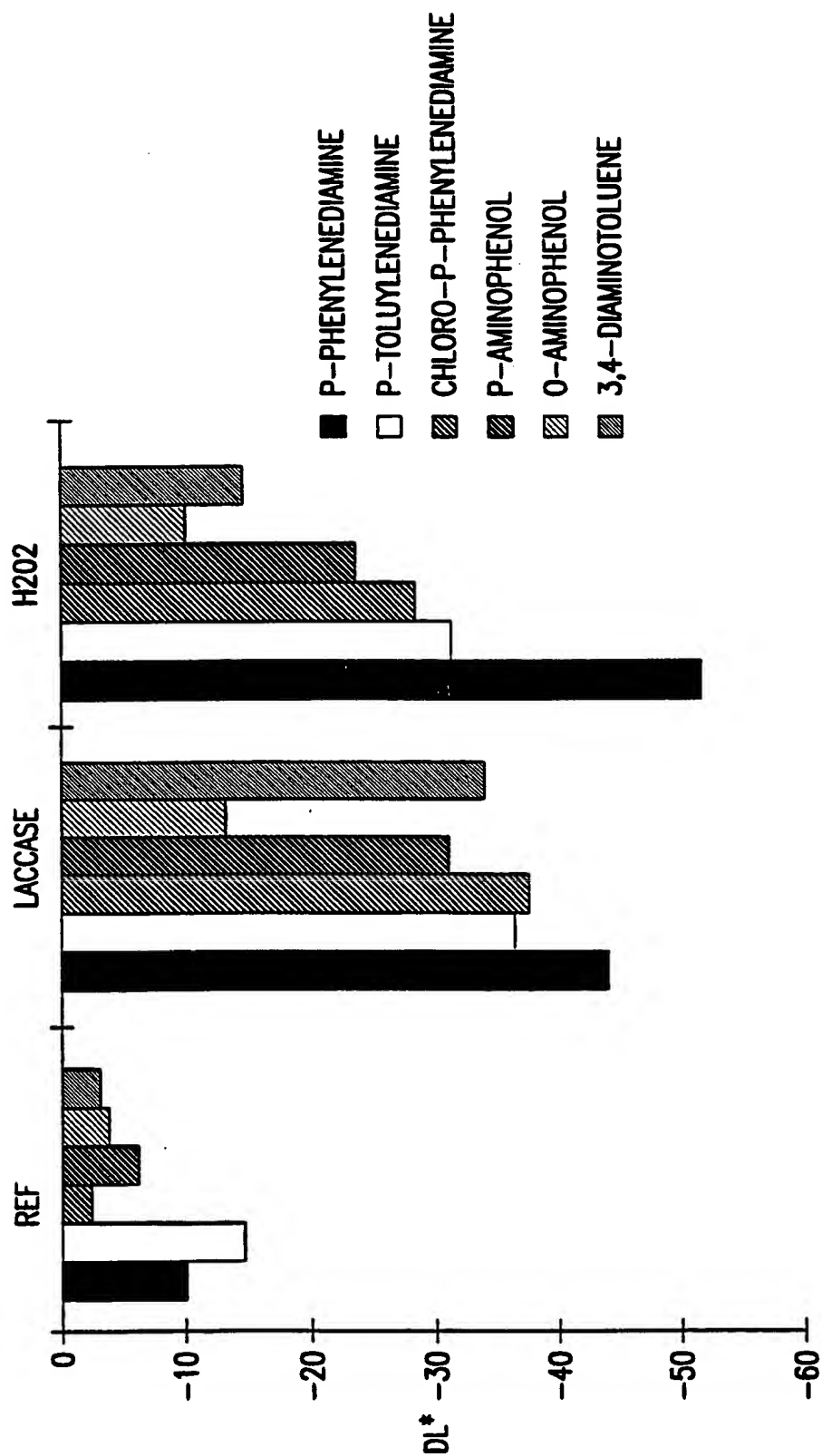


FIG.10

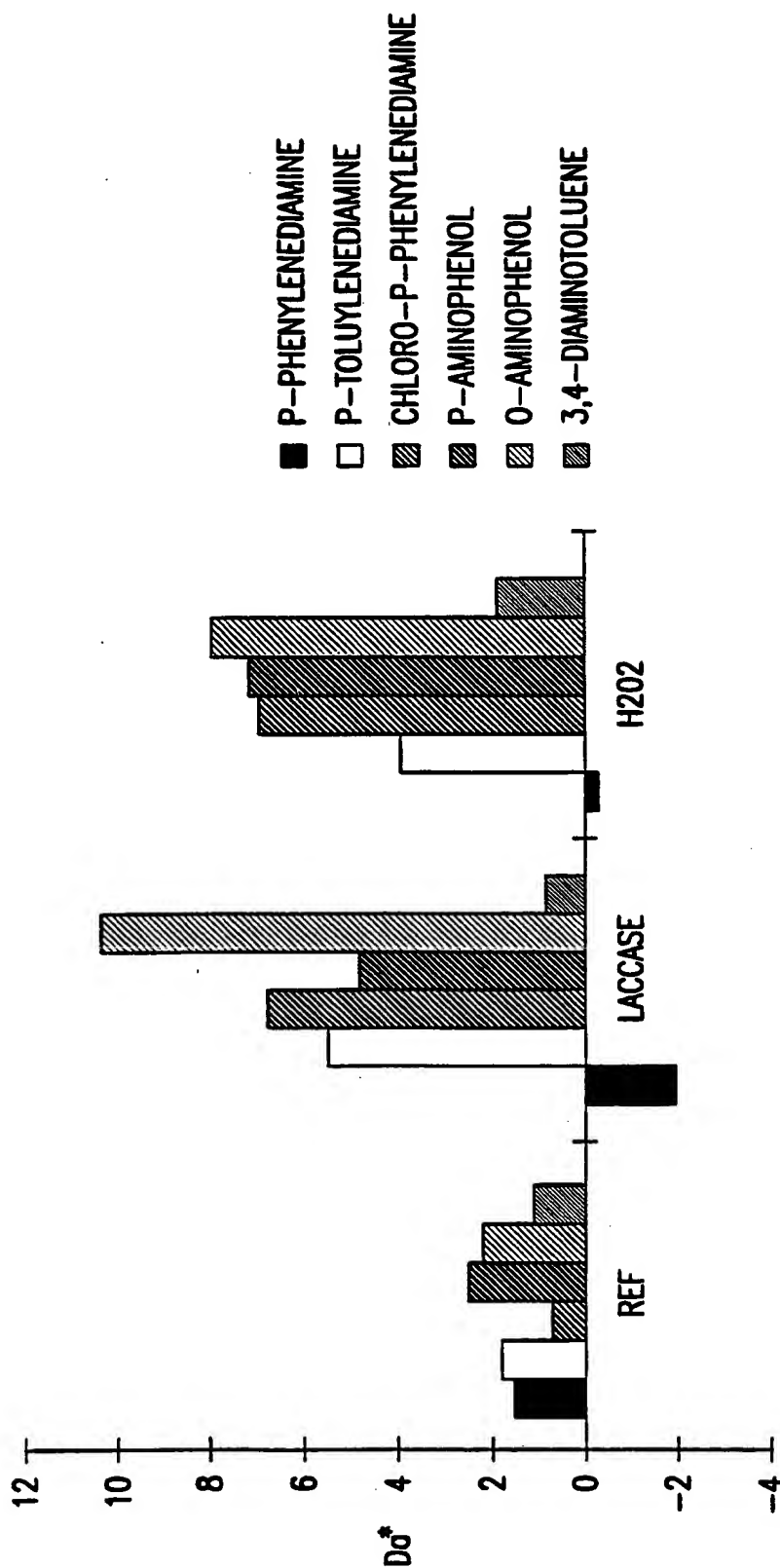


FIG.11

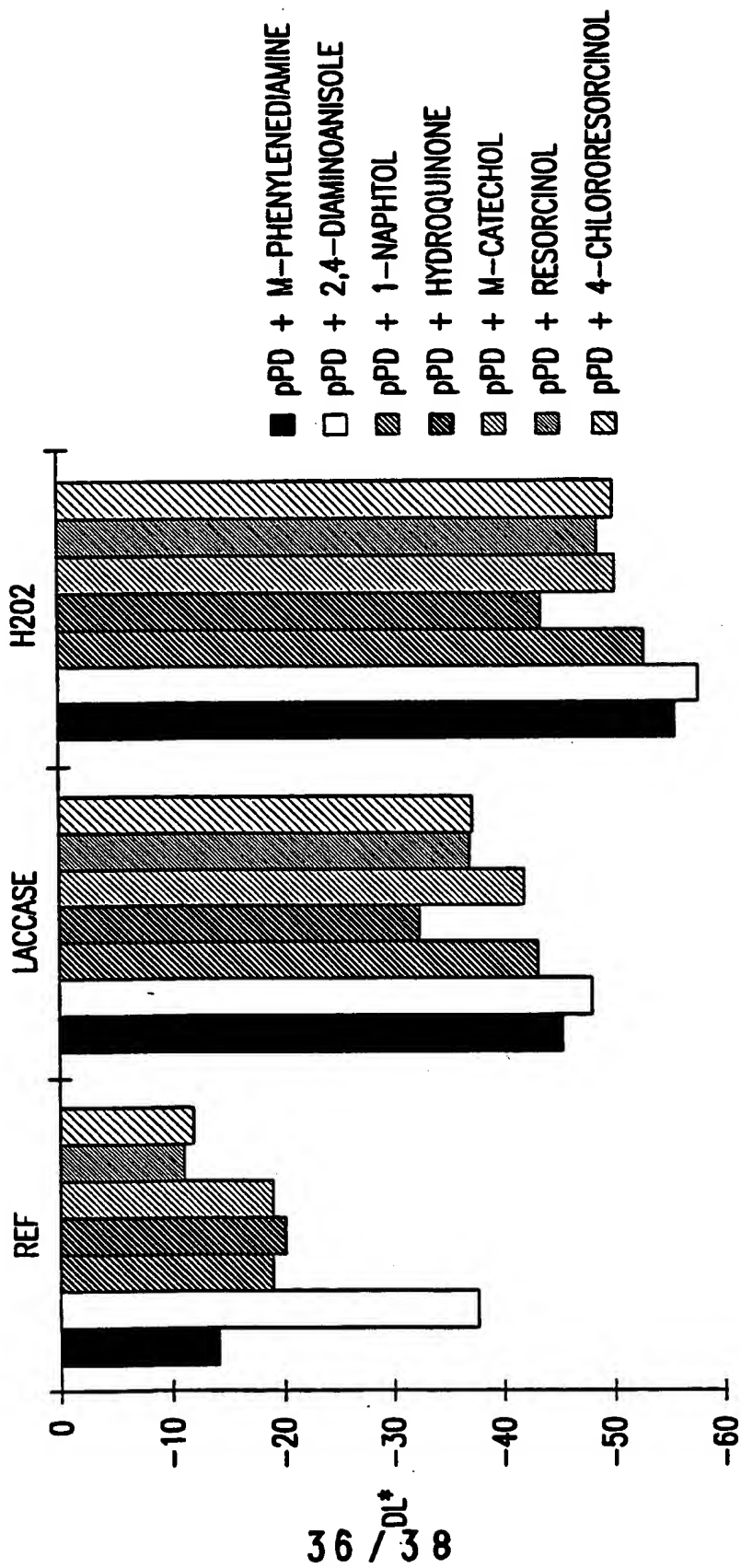


FIG.12



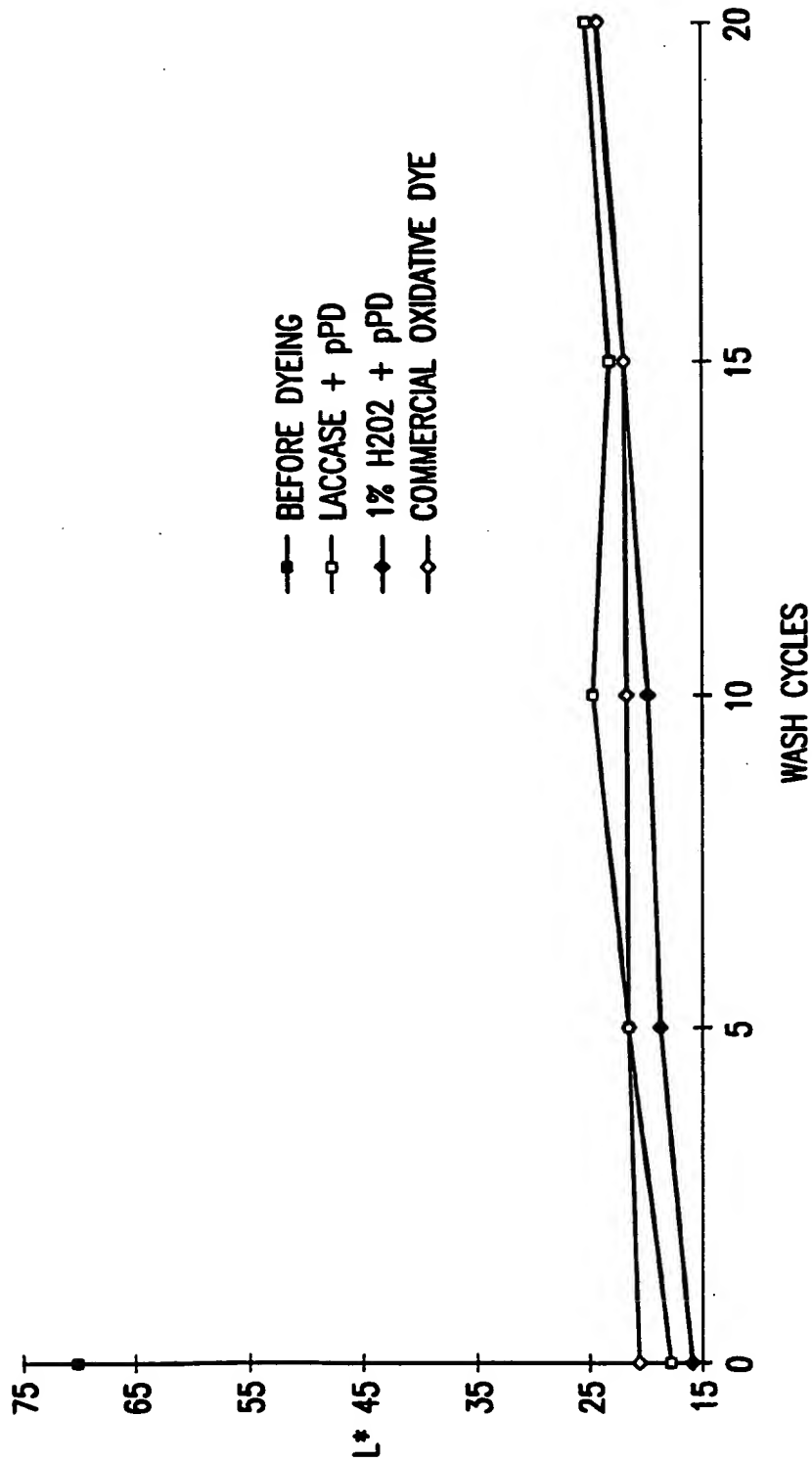


FIG.13

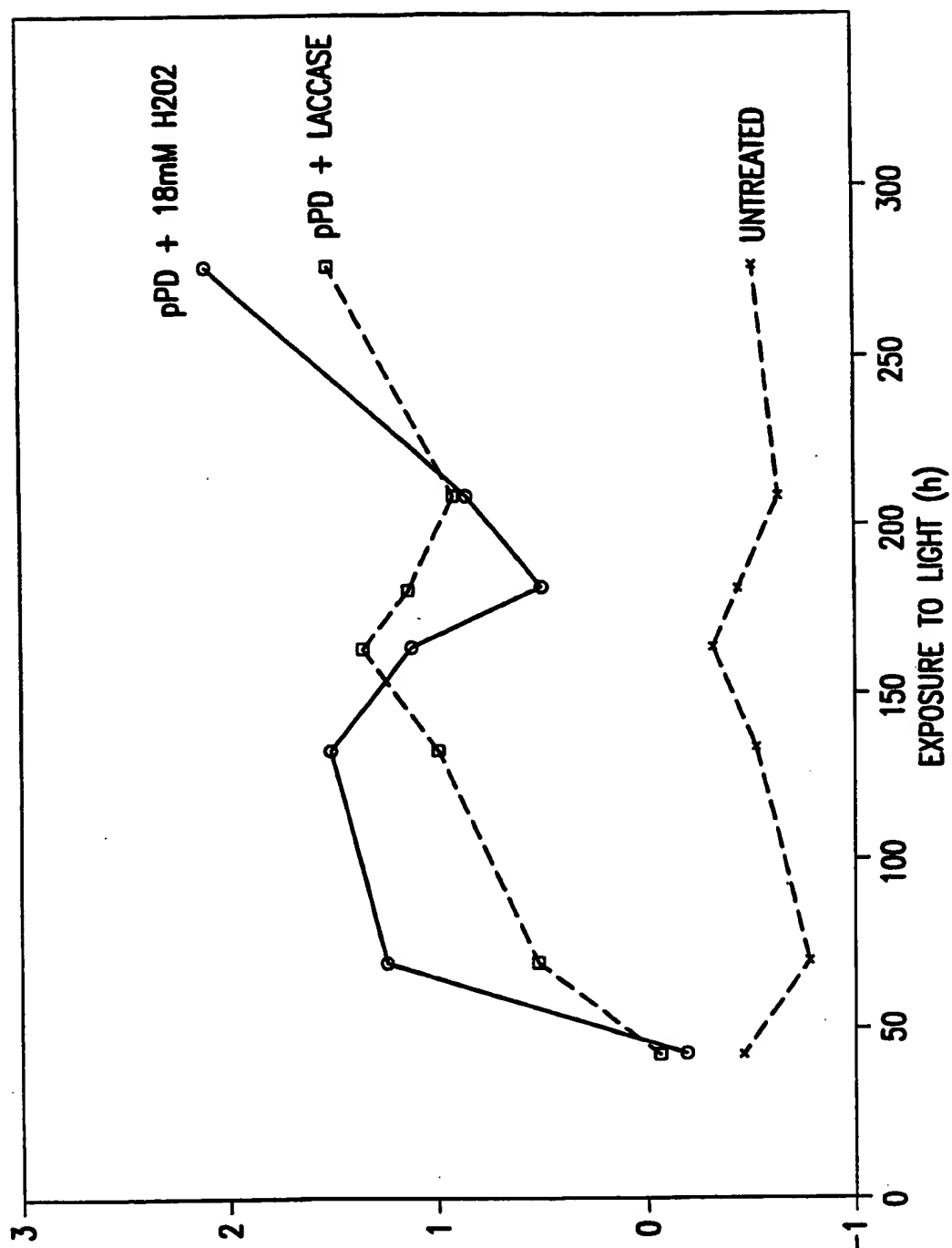


FIG.14

# INTERNATIONAL SEARCH REPORT

Int. Patent Application No  
PCT/US 95/07536

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/53 C12N9/02 C12N1/15 A61K7/13 A61K7/06  
D21C5/00 C12N15/80 //(C12N1/15, C12R1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A61K D21C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GEN. TECH. REP. NC (NORTH CENT. FOR EXP. STN.), vol. 175, 1994 pages 115-118, YAYER D.S. ET AL. 'The molecular cloning and expression of laccase genes from the white-rot basidiomycete Polyporus pinsitu' see the whole document ---	1-48
P,X	WO,A,95 01426 (NOVONORDISK AS ;SCHNEIDER PALLE (DK); PEDERSEN ANDERS HJELHOLT (DK) 12 January 1995 see page 6 - page 7; claim 22; example 2 ---	15-17, 35-41, 45,48
X	DE,C,40 33 246 (PFLEIDERER UNTERNEHMENSVERWALTUNG GMBH & CO.) 27 February 1992 see the whole document ---	15,16,35
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

10 October 1995

Date of mailing of the international search report

09.11.95

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Espen, J

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/07536

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 48, no. 4, 1984 pages 849-854, BOLLAG J.-M. ET AL. 'Comparative studies of extracellular fungal laccases' see page 851; figure 2 ---	15,35
A	DE,C,36 34 761 (HUTTERMANN, A.) 18 February 1988 see the whole document ---	
A	LES COLLOQUES DE L'INRA, vol. 40, 1987 PARIS, pages 223-229, TROJANOWSKI A. ET AL. 'Solubilization and polymerization of lignin by several wood-inhabiting fungi' see the whole document ---	
A	MICROBIOS LETT., vol. 29, no. 113, 1985 pages 37-43, ILAN CHET ET AL. 'Decolourization of the dye Poly B-411 and its correlation with lignin degradation by fungi' see the whole document -----	

**INTERNATIONAL SEARCH REPORT**

Int. onal Application No

**PCT/US 95/07536**

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9501426	12-01-95	AU-B- 6924594	24-01-95
DE-C-4033246	27-02-92	NONE	
DE-C-3634761	18-02-88	EP-A- 0264076	20-04-88